Modulation of Estrogen Signaling in Hepatic and Vascular Tissue

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Modulation of Estrogen Signaling in Hepatic and Vascular tissue

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1. General Introduction

1. Introduction

Atherosclerosis, a pathological process characterized by vascular remodeling, is a leading cause of mortality and morbidity in the western world. Interestingly, atherosclerosis occurs rarely in premenopausal women, but rises sharply after the menopausal transition, when ovarian secretion of sex hormones is low [1-3]. This is associated with an increase in risk factors for atherosclerosis, including dyslipidaemia, insulin resistance, central obesity and hypertension in the postmenopausal period. These observations suggest that female sex steroid hormones provide protection against atherosclerosis in premenopausal women. Indeed, numerous studies have shown an atheroprotective role for estrogens. Estrogens can exert beneficial effects directly on the vessel wall, but they have also been shown to induce favorable effects on serum lipid, glucose and insulin levels [4-6]. Unfortunately, estrogens have also been postulated to induce adverse effects like endometrial cancer, breast cancer, and gallstones [7,8]. In addition, results of the Women's Health Initiative (WHI) trial regarding the vascular effects of hormone replacement therapy (HRT) have shown no demonstrable benefit of HRT [9]. Although some have criticized the design of the WHI study [10], it is also clear that an improved understanding of estrogen action in specific target tissues is required.

This thesis centers on the mechanisms of estrogen action and the effects on the development of atherosclerosis. We have focused on the liver as central organ in lipid and glucose metabolism and the vessel wall as the actual site where the injury occurs. To gain insight in tissue-specific actions of estrogens, we have spent considerable effort to develop tools for liver and blood vessel specific modulation of the estrogen receptor (ER) signaling cascade. The generation, characterization and application of these tools in vitro and in vivo will be described in the different chapters of this thesis.

2. Estrogen action

2.1 Estrogen production

17-β-Estradiol (E_2) is a steroid hormone that is primarily synthesized in the ovary of (premenopausal) women. These hormones function as an endocrine signal by exerting selective effects on distal target tissues. In addition to the female reproductive system, non-reproductive tissues such as the cardiovascular system, the immune system, the central nervous system, bone and brain are target tissues. Thus,

E_2 elicits multiple tissue-specific responses throughout the body, resulting in beneficial but also detrimental responses. In postmenopausal women, systemic E_2 production is ceased and E_2 is no longer able to function as an endocrine factor affecting distal tissues. Nevertheless, both in postmenopausal women and in men, E_2 plays an important physiological role in a number of extragonadal tissues. These tissues, which include adipose tissue, bone, numerous sites in the brain, vascular endothelial and aortic smooth muscle cells, have the capacity to express aromatase. Aromatase cytochrome P450, which is encoded by the *CYP19* gene, catalyzes the biosynthesis of E_2 and thus these tissues are able to produce E_2 themselves. However, E_2 generated via aromatase, acts predominantly at the local tissue level as a paracrine or even intracrine factor in stead of an endocrine factor [11,12]. In addition, in contrast to the ovary, these extragonadal tissues do not contain a full complement of steroidogenic enzymes [13] and therefore are dependent on substrate for aromatase activity on circulating C_{19} androgenic precursors. Because the levels of circulating androgenic precursors are lower in postmenopausal women as compared to the circulating androgenic precursors in men [14], E_2 action is lower in postmenopausal women and thus could accelerate the postmenopausal gender differences.

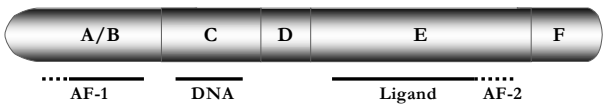


Figure 1. Functional domains of the estrogen receptor

2.2 The Estrogen Receptor

Part of the biological effects of E_2 is mediated through ERs. ERs are members of the steroid/thyroid hormone nuclear receptor superfamily that function as ligand-activated transcription factors [15]. These receptor proteins share a common architecture of six distinct domains designated alphabetically, A-F (Fig. 1) These domains are responsible for ligand binding, DNA binding and transcriptional activation [16-18]. In more detail, the amino terminus (A and B domains) contains a transcriptional activation function (AF-1) that does not require ligand for activity. In stead, it is constitutively active when linked to a suitable DNA-binding domain (DBD) [19]. This linked DBD (C domain) consists of two zinc fingers that recognize specific DNA

sequences, referred as estrogen response elements (EREs) [20]. Next to the DBD, there is a flexible hinge region (D domain) and a ligand binding domain (LBD) (E domain). The ligand binding cavity in association with the carboxy terminal region, which contains a ligand-dependent transcriptional activation domain (AF-2) (F-domain) contributes to transcription activity. Upon ligand binding, conformational changes are induced leading to an interaction surface for cofactors such as steroid receptor coactivator-1 [21]. Maximal activation of ER requires an interaction between the two activation domains AF-1 and AF-2, occurring when ligand and coactivator proteins are present [22].

The different ER domains coordinately regulate ER mediated transcription. In the initially described models, ERs reside in the cytoplasm in complex with heat shock protein 90 (HSP90). Upon ligand binding, ERs dissociate from HSP90, form dimers and interact with EREs within the promoter of their target genes to initiate transcription [23]. However, it is now clear that E_2 action is much more complex than previously thought. ERs not solely function as transcription factors, but also serve as co-activators for other transcription factors. In addition, it seems likely that they have a function outside the nucleus to mediate very rapid cellular responses to E_2. As a consequence, E_2 effects not only depend on the presence of its receptor, but also on the presence and abundance of several interactive proteins that are involved in these different ER pathways. Understanding of these multiple and cross-talking pathways (Fig. 2) in the different E_2 responsive tissues is required for mechanistic insight in the time and tissue-specific effects of E_2.

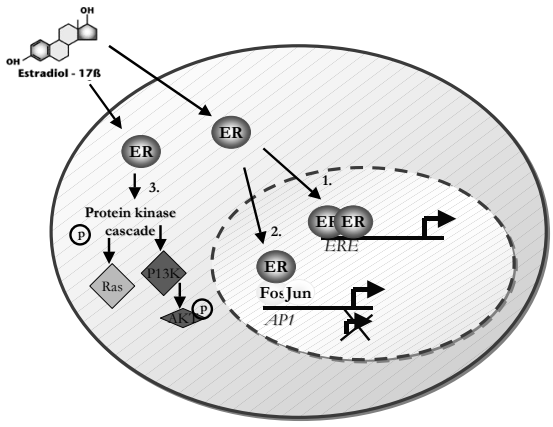


Figure 2. Estrogen receptor (ER) mediated action.
ERs are involved in several independent pathways. **1.** The classical pathway. ER initiates transcription via binding to ERE sites. **2.** The non-classical pathway. ER initiates or represses genes via interfering with other transcription factors. **3.** The non-genomic pathway. ER rapidly induces effects by activating cytoplasmic proteins (phosphorylation)

2.3 Classical ER mediated transcription

The most well-studied pathway of ER action is as ligand activated nuclear transcription factor at classical ERE sites. In this so-called classical mode of ER action, E_2 binding to cytoplasmic ER hormone induces conformational changes in the receptor, which causes dissociation of heat shock proteins that normally maintain the ER in an inactive but activatable configuration. The activated ERs are translocated to the nucleus, homodimerize and bind as dimers to two ERE half-sites that are found within the regulatory regions of their target genes. The conformational changes induced within the LBD allow the recruitment and interaction with basal transcription factors and co-activator proteins, which co-coordinately induce transcription. The ERE binding site has been discovered as a 13-base pair inverted repeat sequence (GGTCAnnnTGACC). However most of the estrogen responsive genes contain non-consensus elements, which exist as single or multiple full or half sites or they contain composite sites, consisting of EREs flanked by response elements for other transcription factors.

2.4 Non-classical ER mediated transcription

It has become apparent that ERs can also mediate transcription via a mechanism that deviates from the classical mode of action. Around one third of the genes in humans that are regulated by ERs do not contain ERE-like sequences [24]. These genes do contain alternative response elements, like AP-1 [25,26], CRE-like elements [27] and USF sites [28], from which ER can also regulate transcription. In this so-called non-classical genomic pathway, ERs do not bind directly to DNA, but modulate the function of other transcription factors through protein-protein interactions with these transcription factors or their co-activators [29]. In this complex, ER functions as a co-activator that stabilizes the DNA binding of the transcription factor complex and/or that recruits other co-activators. Several genes are known to be regulated by E_2 through this non-classical mode of ER action, including, collagenase [30], insulin like growth factor receptor 1 [31] and cyclin D1[32,33].

2.5 Non-genomic ER mediated pathway

Recently, in addition to the well-known genomic effects, E_2 mediated non-transcriptional mechanism of signal transduction have been identified. In these so-

called non-genomic pathways, the effects are very rapid, arising within seconds to few minutes from the challenge with E_2 and frequently involves activation of cytoplasmic or cell membrane bound protein kinases. The E_2 mediated non-genomic actions that have been reported include the mobilization of intracellular calcium [34], the regulation of cell membrane-ion channels [35] and of G-protein-coupled receptors [36] and activation of tyrosine kinases and mitogen activated (MAP) kinases [37]. Evidence that a distinct subpopulation of cell membrane bound ERs exist was already provided in 1977s by Pietras and Szego [38]. However, since the 90's reports have appeared that documented that ERs which were localized at the plasma membrane [39-41] could indeed exert important E_2 mediated cellular effects [42]. With respect to ligand affinity, receptor protein size, and immunological epitopes, the membrane and nuclear ERs are identical. However, since ERs do not have an intrinsic trans-membrane domain [43], the mechanism underlying membrane localization remained unidentified. Recently, it has been discovered in endothelial cells that a subpopulation of ERs is localized to the membrane via interaction with membrane-associated caveolae. Here, E_2 rapidly induces nitric oxide release via a phosphatidylinositol 3-kinase/Akt/endothelial nitric-oxide synthase (eNOS) pathway [44,45]. It has been demonstrated that palmitoylation of ER is required for this ER:protein interaction with caveolin-1 and subsequently for the receptor localization to and maintenance at the plasma membrane.

2.6 Structure of ERα and ERβ

For a long time, studies to unravel E_2 action have focused only on a single ER (nowadays referred as ERα), which was cloned and reported in 1986 [46,47]. However in 1996 a second ER, ERβ, was found [48-51].

Despite the high homology between ERα and ERβ, there is accumulating evidence that the two receptors function differently leading to distinct biological activities. These differences include, for instance, lower transcriptional activity of E_2-bound ERβ on ERE containing promoters [52,53], higher binding affinity of ERβ for the phytoestrogens coumestrol and genistein [54] and opposite actions on gene transcription, as has been observed in response to E_2 and raloxifene at AP-1 sites [55]. Molecular mechanisms for such transcriptional differences are poorly understood, but studies characterizing the structure and function relationships between the ER

subtypes have provided a molecular basis for at least some of their differential transcriptional activities. The DBD and to a lesser extent the LBD of ERα and ERβ exhibit a high degree of homology (96% and 58% amino acid identity, respectively) [56]. Likewise, functions associated with these structural domains such as ERE binding, dimerization, but also affinity to the natural estrogen E_2 are very similar for ERα and ERβ [57-60]. However, as a consequence of reduced homology in the LBD, ligands exhibiting different affinities for ERα and ERβ have also been reported [61,62]. These ligands induce ER subtype specific changes [63,64] resulting in recruitment of diverse co-activators and co-repressors. For example, affinity of ERα for SRC-3 is much higher than that observed for ERβ [65]. Thus the LBD is at least partly involved in mediating ER subtype specific actions. The amino-terminal domain, exhibiting the AF-1 region, is poorly conserved between ERα and ERβ and thus may play a significant additional role in mediating their different transcriptional activation properties. Indeed several studies provided evidence for an important role of the AF-1 region. For instance, amino-terminal deletion of the AF-1 region in ERα led to a loss of transcriptional activity induced via the classical mode of action, whereas amino-terminal deletion in ERβ resulted in an increased transcriptional activity [66]. Thus, ERα and ERβ have different transcriptional activation properties that could result at least in part from structurally divergent LBD and amino-terminal domains.

2.7 Tissue expression pattern ERα and ERβ

Since ERα and ERβ have distinct transcriptional abilities, which could even be opposite to each other, their tissue specific expression pattern is a determinant of the E_2 mediated effects. Both ERs are widely distributed throughout the body. ERα is expressed primarily in the uterus, liver, kidney, and heart, whereas ERβ is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder and central nervous systems. Tissues, which express both ERα and ERβ, are the mammary gland, the adrenals, bone, adipose tissue, vascular endothelium and smooth muscle cells and regions of the brain. In these tissues, there is a potential interplay between the two ERs, and thus their balance is important. For certain genes it has been found that ERβ exhibits an inhibitory activity on ERα-mediated gene expression [67-69]. It remains

to be seen whether this ERβ-dependent antagonism of ERα responses is restricted to a limited number of genes or that it represent a general mechanism in ER signaling.

3. Modulation of estrogen action

3.1 Mouse models

Mice are used as experimental models, because they are small, relatively easy to handle, have a short generation time, and, the strains are genetically defined, which reduces genetic noise. In addition, animal studies allow direct access to tissues for histological and molecular analyses. Thus, although results from mice models cannot always be extrapolated directly to humans, they provide unique mechanistic insight in the actions of E_2 and the role of the ERs.

To explore E_2 signaling, surgical and/or pharmacological manipulations, like ovariectomy (ovx) and systemic administration of estrogenic compounds have been done. Additional insight into the underlying molecular pathway of E_2 action has been obtained from ER knockout and transgenic mouse models. These models include ERα knockout ($ER\alpha^{-/-}$), $ER\beta^{-/-}$ and $ER\alpha/\beta^{-/-}$ double knockout mice [70-73] and aromatase deficient mice (ArKO) [74,75]. Of the $ER\alpha^{-/-}$ mice, two separate lines have been generated, which displayed remarkably different phenotypes. The first generated $ER\alpha^{-/-}$ mice line carries a Neo cassette in exon 1, hereafter designated as $ER\alpha_{neo}^{-/-}$ mice [76]. In these mice, the reproductive function is abolished, but several other effects of estrogen, such as estrogen induced uterine hypertrophy, persist. The persistency of these estrogenic effects is caused by the presence of a chimeric ERα protein of 55 kDa (ERα55). This chimeric ERα is able to exert transcriptional activity, although reduced when compared with the WT full-length ERα66 [77-79]. Thus, precaution has to be taken with interpretation of the data obtained using this mouse model. The second mouse line deficient in ERα was generated in 2000 by deletion of exon 2 [80], designated as $ER\alpha_{\Delta 2}^{-/-}$. These mice displayed a complete and unambiguous inactivation of ERα. Some caution has to be taken with the interpretation of data from this mouse model too, since $ER\alpha_{\Delta 2}^{-/-}$ female mice have approximately 10-fold higher levels of estrogen and also increased testosterone levels as compared to their wt counterparts [81]. In addition, a $ER\beta^{-/-}$ mouse line has been generated [82]. Those appear to have a quite normal phenotype, in which ERβ

deficiency did not affect circulating estrogen and testosterone levels. And although litter size is slightly reduced, they are able to reproduce [83].

Estrogen deficient mice have been generated by disruption of the Cyp19 gene (ArKo mice). Since they lack a functional aromatase enzyme [84], plasma E_2 levels are undetectable. Interestingly, both male and female ArKO mice have elevated plasma levels of testosterone and the luteinizing hormone, which should be taken into account when interpreting data obtained with this model.

Overall, the knockout mouse models have proven to be useful, providing valuable information about E_2 action and the nuclear receptors involved. However, insight in cell and tissue specific actions of E2 in relation to vascular disease is relatively sparse.

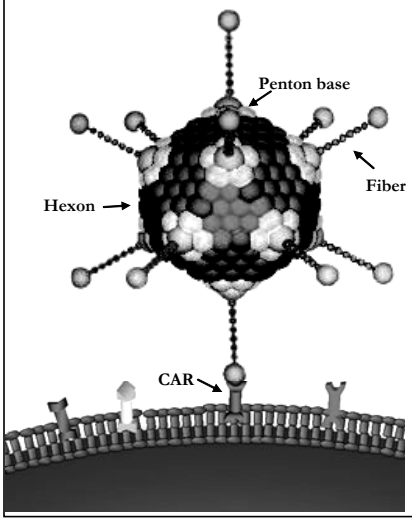
3.2 Gene transfer into liver and the vascular system

An effective strategy to modulate gene expression is by means of adenovirus (Ad) mediated gene transfer. Both wild type and constitutive active or dominant negative variants of the estrogen receptor can be delivered using Ad vectors. In general, the liver is the easiest target to accomplish gene transfer in vivo. The main reason for efficient hepatic gene transfer is the presence of a fenestrated endothelium of 100 nm width that covers the hepatic sinusoids. Consequently, macromolecules such as viral particles that are injected in the blood circulation can cross the endothelium and reach hepatocytes effortless. In addition, hepatic blood flow represents one-fifth of the cardiac output. In contrast, systemic application of vectors to deliver full-length or mutated ERs to vascular tissue is more difficult. On the one hand, treatment efficacy is decreased because vectors are sequestered by liver. On the other hand, the endothelium is refractory to transduction and forms a tight non fenestrated barrier for the underlying vascular smooth muscle cell (VSMC) layer. Thus, introduction of genes to vascular cells in vivo remains a major challenge for current gene therapy strategies.

3.2.1 Adenoviral vectors

Ad vectors are a highly efficient tool for hepatic gene transfer [85,86] and are a commonly used vector for gene delivery to the vascular system. These vectors are generated from human adenovirus serotype 5, which are non-enveloped icosahedral DNA viruses of about 90-nm diameter that can cause infections of the respiratory tracts in humans. The particle is composed of an outer capsid that contains three major components, the hexon, penton base and fiber (Fig 3). The protruding fibers consist of a knob that has a high affinity towards the coxsackie adenovirus receptor (CAR) and thus docks the particle to CAR expressing host cells [87-89]. After this initial binding, the RGD motifs on the penton base interact with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, which leads to clathrin-mediated endocytosis of the virus particle [90-92]. Once endocytosed, the virus escapes the endosome to enter the nucleus. Once the virus has passed its genome to the nucleus, selective transcription and translation are initiated. First, the virus modulates the function of the host cell to facilitate its replication, transcription and translation of the viral genome. Then, the newly synthesized viral components are assembled into new viral particles, which will be released upon cell lysis. These can then initiate a new round of infection and viral replication.

Figure 3. Adenovirus Morphology



To use Ad5 as a delivery device, recombinant Ad vectors have been rendered replication-deficient and less immunogenic by removing the E1 and E3 regions. These regions are essential for the activation of replication of the viral genome and the initiation of a host immune response, respectively. The essential E1 functions are complemented in trans by means of specific cell lines that constitutively express the E1 proteins, such as the 293, 911 and PerC6 cell lines [93,94]. Subsequently, up to 6.5 kb of foreign coding DNA can be introduced into the E1/E3 deleted vector. To transfer the transgene to a particular cell type, the expression pattern of CAR and A5B3 integrins are essential. Although many cell types can be infected with

adenovirus vectors in vitro, for refractory cell types this requires high multiplicities of infection (MOI). High MOI's are associated with cytotoxicity that may interfere with the interpretation of the results.

3.2.2 Targeting adenoviral vectors

Vascular cells express very little, if any CAR and are thus refractory to Ad mediated infection. To improve gene delivery to vascular cells in terms of efficiency (achieve gene transfer to a high percentage of cells with low doses and low immunogenicity) and selectivity (diminish affinity for non-target sites), Ad vectors have been engineered. Two different approaches are used to target transgene expression to alternative non-CAR expressing cells such as endothelial cells (EC) and VSMCs. The first approach modifies the viral capsid through genetic alteration, for example by engineering endothelium-binding peptides into the Ad fiber protein [95,96], or by psuedotyping (exchange of Ad fiber for a fiber from an alternative serotype possessing a more favourable cell binding profile) [97]. The second approach employs bi-valent molecules where one part of the molecule binds to the vector and the other part of the molecule will target the complex to an alternative receptor that is expressed at the surface of the desired target tissue. A commonly used example of a bi-valent molecule is the bispecific antibody [98,99]. In addition to targeting, tissue specific expression can be enhanced by using promoter/enhancer sequences from endothelium- or VSMC-restricted genes [100]. The endothelial specificity of minimal promoters derived from Tie II (angiopoietin receptor), von Willebrand factor, fms-like tyrosine kinase-1, thrombomodulin, E-selectin and ICAM-2 have been demonstrated by transgenic mouse models expressing lacZ driven by these promoters.

4. Estrogen action in the vascular system

4.1 The vessel wall

The vessel wall consists of three well-defined layers: the innermost layer is called the endothelium, the middle layer is called the media, and the outermost layer is known as the adventitia (Fig 4A). Of these three layers, the endothelium is separated from the media by the internal elastic lamina and the media is separated from the adventitia by the external elastic lamina. The endothelium consists of a single contiguous lining of endothelial cells that forms the barrier between the blood

flow and the artery. It has become evident that this endothelium is not a passive barrier. On the contrary, it plays a major role in several processes, including maintaining vascular homeostasis, controlling vascular permeability, inhibiting platelet adhesion and aggregation and limiting activation of the coagulation system. The media consists of VSMC and an extracellular matrix (ECM). The major role of VSMC is to regulate blood pressure and thus blood flow. The outermost layer of the artery, the adventitia, consists of loose matrix of elastin, smooth muscle cells, fibroblasts and collagen.

4.2 Role of estrogen in vascular tone

Vascular tone and function seem to differ between men and women, as women have lower blood pressure than age-matched males [101]. Moreover, hypertension occurs with higher frequency in men and postmenopausal women than in premenopausal women. In part this has been related to the presence of endogenous estrogens, as healthy men treated with aromatase inhibitor displayed impaired vascular dilatation [102,103]. Vascular tone is regulated by a complex set of vasodilator and vasoconstrictor factors that adjust the contractile state of VSMC [104,105]. The endothelium is mainly responsible for the synthesis and secretion of these factors, including angiotensin II, endothelin-1 and NO. In humans, the endothelium-dependent vasodilatory effect of E_2 could at least be partly explained by its enhancement of NO production [106]. Moreover, in vitro studies have confirmed that the endothelial mediated NO release is increased by E_2. This release occurred through both the ERα mediated classical genomic pathway as well as through the rapid non-genomic pathways [107-109]. Recent data have demonstrated that in addition to ERα, ERβ is involved in the regulation of endothelial NO production. Both the ERβ- as well as the ERα-selective agonist, DPN and PPT rapidly induced eNOS activity in EC [110].

The contractile response of the underlying VSMC layer can also be modulated in an endothelium-independent manner. By denudation (stripping of the endothelial layer) of the vessel wall, it has been shown that E_2 is capable of reducing vasoconstriction in an endothelium-independent manner [111]. A predominant role for the E_2 mediated vascular dilatation in endothelial-denuded vessels seemed to be played by ERβ. In mice, ERβ deficiency led to a nearly two-fold enhancement of

phenylephrine -induced vasoconstriction compared to wt controls. In addition, blood pressure was increased in ERβ$^{-/-}$ mice. Inducible NOS (iNOS) appears to be involved in ERβ mediated vascular dilatation. In E_2 treated denudated vessels, enhanced expression of iNOS protein was detected [112,113], whereas reduced iNOS protein levels was observed in aorta of ERα$_{neo}^{-/-}$/ERβ$^{-/-}$ mice [114]. The effect of ERβ on iNOS expression seems to be induced by VSMCs, as demonstrated by an in vitro iNOS promoter study [115]. Overall, E_2 induced stimulation of endothelium dependent and independent vascular relaxation may contribute to the observed gender differences in vascular tone. Depending on the vascular cell type, ERα and ERβ seem to have opposite effects and/or could exert subtype specific effects.

4.3 Role of estrogen in vascular injury

An intact vascular endothelium is critical to the maintenance of normal arterial tone and provides an anti-inflammatory, anti-coagulatory surface. In the case of injury of the endothelium, caused by a wide range of genetic and environmental factors like elevated levels of LDL cholesterol, obesity, diabetes mellitus, cigarette smoking, and exposure to infectious agents [116], EC-activation and VSMCs proliferation are initiated (Fig 4B). These processes are thought to be the precursor of vascular pathologies, including atherosclerosis and restenosis [117,118].

In mouse models, vascular injury can be obtained by denudation of the carotid artery. In this model E_2 has been demonstrated to inhibit VSMC proliferation [119,120]. To clarify the role of ERs in the protective mechanism of E_2 after vascular injury, both ERα$^{-/-}$ and ERβ$^{-/-}$ mice have been used. In wt as well as in ERα$_{neo}^{-/-}$ and ERβ$^{-/-}$ mice, E_2 still attenuates injury induced VSMC proliferation [121,122]. In contrast, in the follow-up study were ERα$_{\Delta 2}^{-/-}$ mice have been used, E_2 was no longer protective [123]. Thus, ERα is involved in E_2 mediated inhibition of VSMC proliferation after vascular injury and the chimeric ERα present in the aorta of ERα$_{neo}^{-/-}$ mice [124] is sufficient to confer complete protection by E_2. Remarkably, in the absence of E_2, ERα$_{\Delta 2}^{-/-}$ mice displayed significantly smaller vascular injury responses as compared to wt and ERβ$^{-/-}$ mice [107, 108]. This signifies either a potential harmful role for E_2-independent ERα mediated activity in the vascular injury response, or in the absence of E_2, ERβ has a beneficial role, which in wt mice is overshadowed by ERα. Rapid restoration of endothelial integrity and reduction of

endothelial activation has a favorable impact on VSMC proliferation [125,126] and thus potentially could reduce the vascular injury response. The E_2 induced attenuation of the response to injury might be due to enhanced re-endothelialization of the damaged arterial segment. Indeed, by use of wt, $ER\beta^{-/-}$ and $ER\alpha_{\Delta 2}{}^{-/-}$ mice models it has been demonstrated that E_2 accelerates endothelial re-growth via ERα [127]. In general, the ability of the endothelium to renew depends on the migration of surrounding mature EC, but also on the attraction and adhesion of circulating endothelial progenitor cells (EPCs) to the injured region, which then differentiate into endothelial-like cells. E_2 has been shown to increase the number of EPCs in the

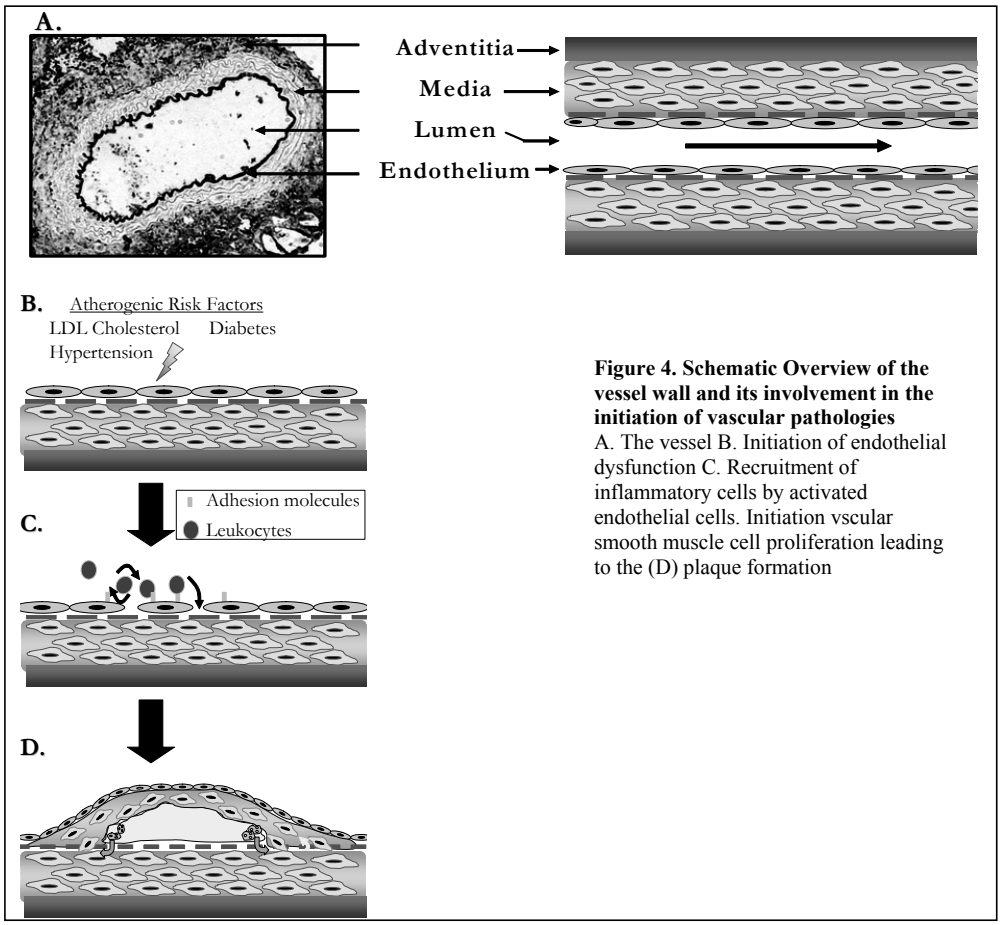


Figure 4. Schematic Overview of the vessel wall and its involvement in the initiation of vascular pathologies
A. The vessel B. Initiation of endothelial dysfunction C. Recruitment of inflammatory cells by activated endothelial cells. Initiation vscular smooth muscle cell proliferation leading to the (D) plaque formation

circulation but also at the site of vascular lesion. As a consequence, the vascular injury response has been reduced [128]. Thus, the protective vascular effects of E_2 are at least partly due to effects on circulating EPCs. Accordingly, the available mouse models of estrogen deficiency provide evidence that E_2 mediated activation of ERα reduces the vascular injury response. However, whether this effect fully accounts on the enhanced attraction and adhesion of circulating EPCs or whether there is also an effect locally at the surrounding mature ECs remains to be addressed.

4.3.1 Atherosclerosis

Vascular injury is an important initial step in the development of atherosclerosis, a progressive disease in which fat and cholesterol are deposited along artery walls (Fig. 4C). In short, due to vascular injury, permeability and expression of endothelial adhesion molecules is enhanced. Consequently, circulating monocytes and lymphocytes interact with the vessel wall. These inflammatory cells secrete cytokines and chemokines (chemoattractive cytokines), which initiate a whole spectrum of reactions leading to vascular smooth muscle cell hyperplasia, intimal migration and further accumulation of lipids. If the damaging insult persists, the inflammatory process may become chronic, the fibro proliferative response persists and lipids continue to accumulate within the vessel wall. Eventually, the enlarged fatty lesion may restrict blood flow through the blood vessel, increasing the risk of heart attack and stroke.

To study the role of E_2 in the pathogenesis of atherosclerosis, atherosclerosis-prone mouse models, including apolipoprotein E (ApoE) knockout and low-density lipoprotein (LDL) receptor (Ldlr) knockout mice have been used. In ovariectomized (ovx) $ApoE^{-/-}$ female mice, systemic administration of E_2 resulted in a consistent and dramatic inhibition of lesion initiation and progression [129-131]. In addition to its inhibitory effect in females, estrogen appears to be equally efficacious in males. For example, Nathan and coworkers [132] have shown that orchidectomy increased lesion size in $Ldlr^{-/-}$ males, which was reversed by exogenous administration of either E_2 or testosterone. Co-administration of an aromatase inhibitor, on the other hand, removed the atheroprotective effect of exogenous testosterone, suggesting that local conversion of testosterone to E_2 in vascular cells attenuates atherosclerosis in male mice. In addition, in the aorta of streptozotocin-induced hyperglycemic $Apoe^{-/-}$ males, E_2

reduced lesion size and prevented calcified cartilaginous metaplasia [133]. The observed E_2 mediated inhibition of lesion size was in some studies associated with a reduction in total plasma cholesterol levels, [134-136], but not in all [137,138]. Thus, E_2 possesses cardiovascular protective actions beyond an effect on plasma lipids, most likely via direct effects on the vessel wall.

The atheroprotective action of E_2 could be established trough ERα and ERβ, as both ERs are present in VSMC and EC. Absolute expression levels of ERs in diverse vascular beds and between the two sexes have not been characterized yet. But, the overall expression level in vascular cells is low. Moreover, ERs are absent from various vascular cells kept in culture, which complicates the analsysis of the role of ERs in the vasculature. Thus far, to investigate the relative contribution of each receptor in the atheroprotective role of E_2, $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice crossbred with $ApoE^{-/-}$ mice have been used. The inhibitory effect of E_2 on atherosclerotic lesion progression obtained in $ApoE^{-/-}$ females was almost completely abrogated in $ER\alpha_{neo}{}^{-/-}$ $Apoe^{-/-}$ mice [139]. In addition, the plasma lipid-lowering effect of E_2 was eliminated. However, fibrous caps and other advanced lesion characteristics were reduced in E_2 treated $ER\alpha_{neo}{}^{-/-}Apoe^{-/-}$ as compared to control $ER\alpha_{neo}{}^{-/-}Apoe^{-/-}$ mice [140]. Probably, this residual protective effect is mediated by the presence of the chimeric ERα protein. Conversely, it has been found that in $ER\beta^{-/-}Apoe^{-/-}$ mice, E_2 treatment inhibited atherosclerotic lesion progression equally as compared to $Apoe^{-/-}$ females. Thus E_2 is fully atheroprotective in the absence of ERβ (reviewed in [141], manuscript data in preparation), demonstrating that at least at early stages of plaque formation, the anti-atherogenic effect of E_2 is primarily mediated through ERα and independent of ERβ.

4.3.2 Restenosis

Occlusion of the artery, as occurs in atherosclerotic vessels, can be mechanically treated. The most commonly used therapy of atherosclerotic complications consists of percutaneous transluminal coronary angioplasty (PTCA) followed by endovascular stent implantation [142]. This procedure depends on a catheter containing a deflated balloon. Once the catheter is passed into the narrowed part of the artery, the balloon is inflated allowing more blood flow. The immediate results are good, but as a consequence of constrictive remodeling and formation of a

neointima rich in proliferating SMC and ECM, restenosis occurs within a few months in 30–50% of treated patients. An implanted stent, a spring-like device designed to push open the artery, can prevent constrictive remodeling. However, neointimal proliferation still occurs and is responsible for restenosis in 20–30% of the stent-treated patients. [143,144]. Currently, to prevent intrastent restenosis, stents have been coated with the anti-mitotic drug, Rapamycine or Taxol, which seems very efficient in preventing neointimal hyperplasia. However, these drugs also inhibit the re-endothelialization process, as was demonstrated in large animal models [145].

There is currently considerable attention for drugs that favor re-endothelialization, including drugs that act on the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-1 or –2. However, these drugs have failed due to pleiotropic and deleterious effects. Within this context E_2 has also been considered. The vascular injury models have already demonstrated its anti-mitotic and endothelial re-growth properties [146,147]. In addition, pig models have been used, which displayed improved endothelial function, enhanced re-endothelialization and decreased neointima formation after intra-muscular injections of E_2 during stenting [148], but also after local delivery of E_2 during percutaneous transluminal coronary angioplasty and stenting [149-151]. In humans, a pilot study with E_2-eluting stents has been performed, which did not demonstrate deleterious affects [152]. A randomized follow-up study is required to fully evaluate the potential benefit of E2-coated stents. At the moment, the underlying mechanism and the subsequent involvement of ERα and ERβ are unknown and receptor-specific ligands may have differential effects.

5. Estrogen and Lipid & Glucose Metabolism

5.1 Lipid & glucose metabolism

Hyperlipidemia and insulin resitance are major risk factors for the development of cardiovascular disease.The body has developed a sophisticated lipoprotein and glucose transport system to meet to the diverse demands from different tissues under different conditions. These two systems are heavily interconnected and excess energy intake or genetic defects can deregulate lipid and glucose metabolism, leading to hyperlipidemia and insulin resistance and increased risk for cardiovascular disease. Insulin resistance is characterized by a diminished biological effect of insulin on glucose and free fatty acid (FFA) uptake by skeletal

muscle and adipose tissue, respectively and the suppression of glucose output by the liver (via decreased glyconeogenesis and glycogenolysis).

The liver forms the central site of lipid and glucose metabolism and therefore, plays an essential role in the maintenance of whole body energy homeostasis. It removes remnant lipoproteins from, and delivers newly synthesized lipoproteins to the bloodstream. To maintain the fairly steady concentration of glucose in the blood, the liver takes up and releases glucose into the bloodstream. Furthermore, it expresses a well-orchestrated network of genes that maintain the intra-hepatic cholesterol and glucose homeostasis. It is the main organ involved in de novo FA synthesis. Newly synthesized FA can be converted into triglycerides (TG) to be stored or secreted as VLDL-TG. FA can also be used for energy production via β-oxidation. Glucose can be produced directly through gluconeogenesis from non-carbohydrate sources like amino acids, glycerol and lactate. The liver is also able to produce glucose through phosphorylation of glycogen, the storage form of glucose. This process is called glycogenolysis. On the other hand, when blood glucose levels are high, the liver will function as reservoir to take up and convert the excess of glucose into glycogen for future needs.

5.2 Effects of estrogen on lipid and glucose metabolism

Estrogens seem to be implicated in whole body energy homeostasis. Both gender and menopausal status influence lipid and glucose metabolism [153-155]. For example, menopause is associated with lipid abnormalities. Moreover, menopause is also associated with fat accumulation in the abdominal regions, which again is associated with increased plasma FFA and decreased adiponectin levels, both important components of the insulin-resistance syndrome [156,157]. The importance of estrogens has been revealed by individuals that carry mutations in the gene encoding aromatase. They develop obesity, insulin resistance, hypercholesterolemia, and hypertriglyceridemia [158-162]. Models of estrogen deficiency have been used to obtain more insight. ArKO mice age-progressively develop hypercholesterolemia, hyperleptinemia, and become obese. By 1 yr of age, ArKO males also exhibit elevated plasma triglyceride levels and develop hepatic steatosis [163]. MRI data of ArKO mice reveal that females have three times and males have twice as much adipose tissue as compared to wt mice. ER deficient models have highlighted the importance

of ERα and ERβ in lipid and glucose metabolism. Both ERα$^{-/-}$ and ERα/ERβ$^{-/-}$ mice develop a lipid phenotype similar to the ArKOs, whereas no lipid phenotype is described in ERβ$^{-/-}$ mice [164,165]. ERα deficiency also results in insulin resistance, glucose intolerance, and adipose hyperplasia and hypertrophy in both sexes, as studied in ERα$_{neo}^{-/-}$ [166]. This seems to be comparable with the human situation. One adult male with ERα deficiency has been described [167] and the clinical features of this patient include glucose intolerance, hyperinsulinemia, and lipid abnormalities [168,169]. Interestingly, a role of ERβ was indicated by estrogen depletion (ovx) and exogenous E_2 treatment of ERα$_{neo}^{-/-}$ mice. These experiments demonstrated that removing of the E_2/ERβ signaling cascade by ovx resulted in reduced body and fat-pad weights and adipose size, which could be reversed by E_2 treatment. This indicates that ERβ mediates effects on adipose tissue that are opposite to those of ERα [170]. In addition, estrogen depletion of ERα$_{neo}^{-/-}$ mice improved glucose tolerance and insulin sensitivity, suggesting a harmful role for ERβ in glucose metabolism. Thus, a clear physiological role in the regulation of lipoprotein metabolism in mice has been ascribed to ERα, whereas both ERα and ERβ influence glucose metabolism. However, it should be mentioned that ERα most likely plays the most dominant role in glucose metabolism, since thus far the role of ERβ is only apparent under ERα deficient conditions.

5.3 Role of estrogen in the liver

In the liver, estrogens can enhance liver regeneration and suppress liver fibrosis [171,172]. However, the involvement of estrogens in the hepatic lipid and glucose signaling cascade is less clear. Relatively few reports have appeared in the literature, focusing on hepatic lipid and glucose regulated genes. Of the two ERs only ERα is expressed in liver [173-175], which is in accordance with the fact that ERα seems to play a more important role in lipid metabolism than ERβ [176,177]. The involvement of estrogens and ERα in the regulation of intra-hepatic lipid levels has been demonstrated in ArKO, ERα$_{neo}^{-/-}$ and ERα$_{\Delta 2}^{-/-}$ mice. In all these models analysis of their hepatic lipid content revealed a 3- to 5-fold increase in the TG level [178,179]. In addition, 6 weeks of E_2 treatment in ArKO males effectively blocked the development of hepatic steatosis. Molecular characterization of ArKO mice revealed

that the intra-hepatic signaling pathway was disturbed towards a situation of both enhanced input (enzymes involved in fatty acid synthesis were increased) as well as reduced output (enzymes involved β-oxidation were decreased). These data demonstrate that estrogens do seem to play an important role in hepatic lipid and carbohydrate metabolism, however because the hepatic lipid phenotype in the ArKO and ERα$^{-/-}$ mice is still sex dependent, it seems likely that estrogens are not the sole determinant of the gender-related differences.

A small number of studies have gained more insights in the (direct) effect of estrogens on hepatic genes regulating glucose and lipid homeostasis. The orphan short heterodimer partner (SHP) appears to be induced by chronic [180], but also instant administration of estrogen [181] in liver of wt mice. However, induction of SHP did not inhibit expression of the known SHP target genes cholesterol 7α-hydroxylase (CYP7A1) or sterol 12α- hydroxylase (CYP8B1) and thus the biological implication of estrogen induced expression of hepatic SHP remains to be determined. SR-BI and SR-BII are both HDL receptors involved in the internalization of HDL cholesterol esters, with SR-BII being approximately 4-fold less efficient than SR-BI. Rat studies have found E_2 mediated regulation of hepatic SR-BI and SRBII expression levels [182,183]. However, the underlying mechanism and its impact on HDL metabolism is unclear. Hepatic lipase (HL) participates in the uptake of HDL particles by hepatocytes. E_2 has been shown to increase HL mRNA as well as HL activity with the concomitant lowering of plasma levels of HDL [184]. Apo A-I is the major protein constituent of HDL and has been attributed to its cardioprotective effect [185,186]. Estrogens have been shown to induce Apo A-I promoter activity and gene expression [187-189]. In summary, E_2 clearly affects lipid and glucose metabolism. Although some studies have reported hepatic lipid target genes, the role of liver is not thoroughly known.

6. Thesis Outline

In this thesis we have addressed the role of estrogen signaling in liver and vessel wall with emphasis on the link with vascular disease. To study E_2 signaling in selected tissues, we set out to develop tools to modulate the E_2 signaling cascade in a tissue-specific manner. In chapters 2-4, we have focused on the liver and addressed the physiology of estrogen signaling in the development of metabolic disorders. In

chapter 2, we have generated short interfering RNA constructs to down-regulate mouse ERα mRNA levels. By producing Ad vectors expressing shRNA against mERα (Ad.shERα), we generated a model to study the role of hepatic ER signaling. Both hepatic ERα RNA levels, as well as hepatic ERα activity were monitored in time and found to be significantly decreased. The Ad.shERα is further explored in **chapter 3**, in which the effect of hepatic ERα repression on lipid metabolism has been analyzed. Ad.shERα was intravenously injected in APOE*3-Leiden mice, a mouse model for hyperlipidemia. After several days, when hepatic ERα RNA and protein levels were significantly down-regulated, hepatic VLDL-TG production, lipid levels, and mRNA levels of relevant lipid-related genes were analyzed. Surprisingly, we found that the hepatic ERα levels are not a limiting factor in lipid metabolism. In **chapter 4**, we have studied the acute effect of E_2 on insulin sensitivity. Although E_2 was applied systemically, we found by using a sophisticated in vivo imaging setup that exclusive and maximal activation of hepatic ER was achieved six hours after E_2 administration. Taken into account that the effects were examined after this short period of time, this study provides evidence for a role for hepatic ERα in maintaining glucose homeostasis.

In chapters 5-8 of this thesis, we set out to develop models to modulate estrogen signaling in the vessel wall. In **chapter 5**, Ad vectors have been targeted to enhance gene transfer to transformed as well as to primary vascular cells. The targeting approach is based on a bi-functional linker construct, which contains the extra cellular domain of the Ad receptor linked to a cRGD peptide. This resulted in a targeting construct that binds to the Ad vector at one side and to $\alpha_v\beta_{3/5}$ integrins at the other site. Both primary as well as transformed vascular cells were infected with a high efficiency using this construct. In a subsequent study, we set out to target Ad vectors to the carotid artery of mice in vivo. **Chapter 6** describes the work that has been performed to obtain vascular gene transfer in vivo. Although de-targeting of the liver was achieved successfully, targeting using two independent ligands failed to redirect tropism of the Ad vectors. Experiments indicate that stability of Ad in the circulation may be an important limitation. In **chapter 7**, the effect of E_2 on the expression of adhesion molecules in EC in presence of normal and reduced ERα levels has been analyzed. In this study, we have generated shERα expressing lentiviral vectors that result in persistent reduction of ERα levels. These data

demonstrate that E_2 reduces the expression of adhesion factors, suggesting an anti-inflammatory role for E_2. In this response, ERα is required but not a rate limiting factor. **In chapter 8,** we evaluated the specific role of ERα and ERβ in the vascular wall in vivo. A non-constrictive drug-eluting collar was placed around the femoral artery of mice, which simultaneously induces intimal proliferation and releases either placebo, ERα or ERβ specific agonists. These data demonstrated that in adition to ERα, ERβ is able to inhibit neointima formation. In the last chapter, **chapter 9**, the findings presented in this thesis and possibilities for future research are discussed.

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2.

Efficient *in vivo* knock-down of estrogen receptor alpha: application of recombinant adenovirus vectors for delivery of short hairpin RNA

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Abstract

Background*:* Adenovirus (Ad) mediated gene transfer is a well-established tool to transiently express constructs in livers of mice *in vivo*. In the present study, we determined the specificity and efficiency of Ad vectors expressing short hairpin (sh) RNA constructs to knock-down the estrogen receptor α (ERα). ***Results****:* Two different shRNA constructs derived from the murine ERα coding sequence were designed (shERα). *In vitro*, transfection of three mouse cell lines with pSUPER-shERα constructs resulted in up to 80% reduction of endogenous ERα activity. A single mismatch in the target sequence eliminated the reduction of ERα activity, demonstrating the specificity of shERα. The subsequently generated Ad.shERα vectors were equally effective *in vitro*. *In vivo*, intravenous administration of Ad.shERα resulted in 70% reduced hepatic mouse ERα mRNA levels. Co-injection of Ad.shERα with an Ad vector containing a luciferase (luc) gene driven by an estrogen responsive element (ERE) containing promoter resulted in a significant (90% on day five) down-regulation of hepatic luciferase activity, as determined by non-invasive optical imaging. Down-regulation was sustained up to day seven post-injection. ***Conclusion:*** Ad mediated transfer of shERα expression constructs results in efficient and specific knockdown of endogenous ERα transcription both *in vitro* and *in vivo*.

Introduction

Estrogen exerts various biological effects in numerous organs throughout the body and has been implicated in the pathophysiology of a number of diseases including breast cancer, osteoporosis and cardiovascular disorders. Most of the estrogenic effects are mediated via the two known estrogen receptors, ERα and ERβ. These estrogen receptors are ligand-dependent transcription factors that can modulate gene transcription directly but also indirectly. Thus far, there is a relative paucity in the description of the role of estrogen and estrogen receptors in specific organs. Most studies have been performed using non-tissue specific manipulation of ER signaling such as complete knockouts either via deletion of the estrogen receptor or via deletion of estrogen production by ovariectomy. The availability of tools to specifically address the role of ER signaling in individual tissues would thus fill a void.

Short synthetic duplexes of 21 nucleotides long RNA molecules can specifically inhibit gene expression in mammalian cells [1]. Because of their efficacy and specificity, siRNA molecules provide a powerful tool to dissect gene function. To expand the applicability of the siRNA approach, Brummelkamp and co-workers [2] have introduced

vector-based siRNA expression systems. By directing the synthesis of shRNA via the polymerase-III H1 RNA gene promoter, effective siRNA molecules are formed intracellular after transfection of shRNA expression constructs. To further expand the applicability of the siRNA approach, recombinant retro- and adenoviral based vectors have been designed [3,4]. Of these, adenoviral vectors offer the advantage of highly efficient infection of a broad range of cells, independent of active cell division. Moreover, high titers can be obtained and intravenous injection results in efficient transduction of the liver.

The present study was designed to generate tools to address the role of ERα in a tissue- and time- specific manner. To this end, we have developed recombinant Ad vectors encoding shRNA's directed against mouse ERα (Ad.shERα). Introduction of shERα, either by transfection or by Ad mediated gene transfer into different murine cell lines, led to efficient sequence specific repression of ER mediated transcription. Furthermore, intravenously administration of Ad.shERα resulted in efficient reduction of hepatic ERα mRNA levels ($P < 0.005$) and ERα functionality.

Results

Efficient and specific knock-down of endogenous mERα *in vitro*: Transfection with pSUPER-shERα constructs

Three pSUPER-derived vectors [2] designed to drive expression shERα sequences were constructed. Two vectors contained sh sequences derived from the boundary of the DNA binding domain and the hinge region (shERα_1103), or from the ligand binding domain (shERα_1395) of mERα, respectively. A third expression vector contained both the shERα_1103 and shERα_1395 expression cassettes in series (shERα_tandem).

The efficiency of the shERα constructs for reducing endogenous ERα activity *in vitro* was determined using a luciferase reporter assay. For this purpose, the pSUPER-shERα_1395, pSUPER-shERα_1103, or pSUPER-shERα_tandem were transfected together with a reporter plasmid carrying a trimer of ERE plus TATA box upstream of luciferase (pERE-Luc) into endothelial cell lines (EOMA and H5V) and in mouse breast cancer cells (MXT). As shown in Figure 1A, upon transfection with shERα_1395, relative luciferase activity in lysates of all three cell lines was reduced by 70-80%. A similar result was obtained with shERα_1103 in EOMA's. In addition, the shERα_tandem expression construct proved to

be more efficient than either of single shERα contructs alone in the EOMA cells, adding some 15% to the 70% reduction observed with shERα_1395 (Fig. 1A).

To evaluate the specificity of the shERα construct, shERα_1395 was introduced into EOMA cells over-expressing either mouse ERα or human ERα. The ERα_1395 target sequence contains only a single mismatch with the human ERα (Fig. 1B). Significant suppression of ERα mediated transcription was only observed in lysates of cells that were transfected with mouse ERα but not with human ERα (Fig. 1C). Thus, the observed effects of shERα_1395 are specific for mouse ERα. Moreover, changing a single nucleotide in shERα_1395 completely abolished the silencing effect (data not shown). By western blotting, the effect of shERα on ERα protein expression was studied (Fig. 1D). In the presence of shERα_1395, ERα protein levels were reduced to 33% as compared to control transfected cells. This reduction correlated well with our findings in the luciferase reporter assay. Thus, the observed inhibition of luciferase activity upon treatment with shERα_1395 or shERα_1103 is caused by reduced accumulation of mERα protein. All together, the shERα_1395 and shERα_1103 expression vectors are effective and specific in repression of murine ERα expression.

Knock-down of hepatic ERα expression *in vivo*: using Ad.shERα vectors

To repress ERα activity *in vivo*, Ad vectors expressing either shERα_1395 (Ad.shERα_1395), shERα_1103 (Ad.shERα_1103) or both (Ad.shERα_tandem) were generated (Fig. 2A). The H1 RNA promoter plus shERα expression cassettes were sub-cloned from the corresponding pSUPER into pAdTrack [5], which is engineered to co-express GFP enabling the tracking of infected cells. In addition, we constructed a control AdTrack plasmid, carrying only the H1 RNA promoter, which allowed for the generation of Ad.Empty. Prior to the evaluation of recombinant Ad vectors *in vivo*, we tested the functionality of the vectors *in vitro*. EOMA and MXT cells were transfected with pERE-luc, and subsequently infected with Ad.Empty or the Ad.shERα vectors. Fluorescence analysis indicated a near 100% infection percentage. The luciferase experiments (Fig. 2B) were comparable to those obtained with transfection of the pSUPER constructs (Fig. 1A): both Ad.shERα vectors repressed luciferase reporter activity up to 90%. Thus, Ad.shERα vectors were found to be fully functional with respect to repression of mERα activity.

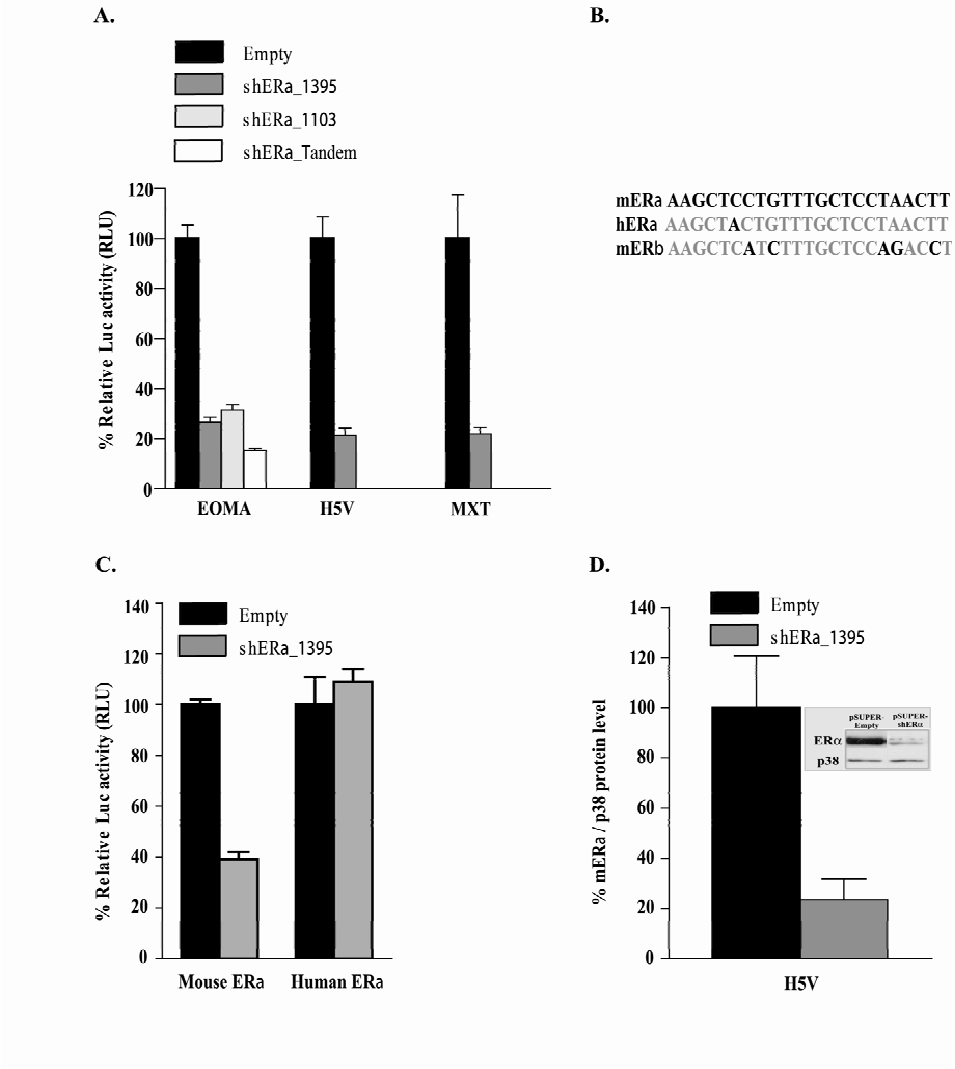


Figure 1. Affectivity and specificity of pSUPER mediated expression of shERα in mouse cell lines
(A+C) The indicated mouse cell lines were co-transfected with, ERE-Luc, CMV-LacZ, and pSUPER-empty, pSUPER-shERα_1395, pSUPER- shERα_1103, or pSUPER- shERα_tandem. Subsequently, the cells were treated 24 hours with 10^{-9} M 17-β-estradiol. Luciferase activity was measured 48 hours after transfection and after correction for LacZ expression, represented as the mean (n=3) ± SD relative to the transfection with pSUPER-empty. (A) Endogenous mouse ERα mediated transcription in EOMA, H5V and MXT cells after introducing pSUPER +/- shERα. (B) The 19-nt target-recognition sequence of ERα_1395 contains one mismatch with human ERα and five mismatches with the mouse ERβ sequence. (C) ERα mediated transcription in EOMA cells after over expression of either mouse ERα- or human ERα-expression vectors in presence of pSUPER empty or pSUPER shERα_1395 (D) Western blot analysis of H5V cells co-transfected with pCMV-mERα and pSUPER-empty or pSUPER-shERα_1395. The lysates were analysed by immunoblotting (insert-photo) with anti-mouse ERα and anti-p38. The intensity of the bands was quantified and normalized to cells transfected with pSUPER-empty. The relative ERα protein levels are presented (bar-diagram) as mean (n=3) +/- SD.

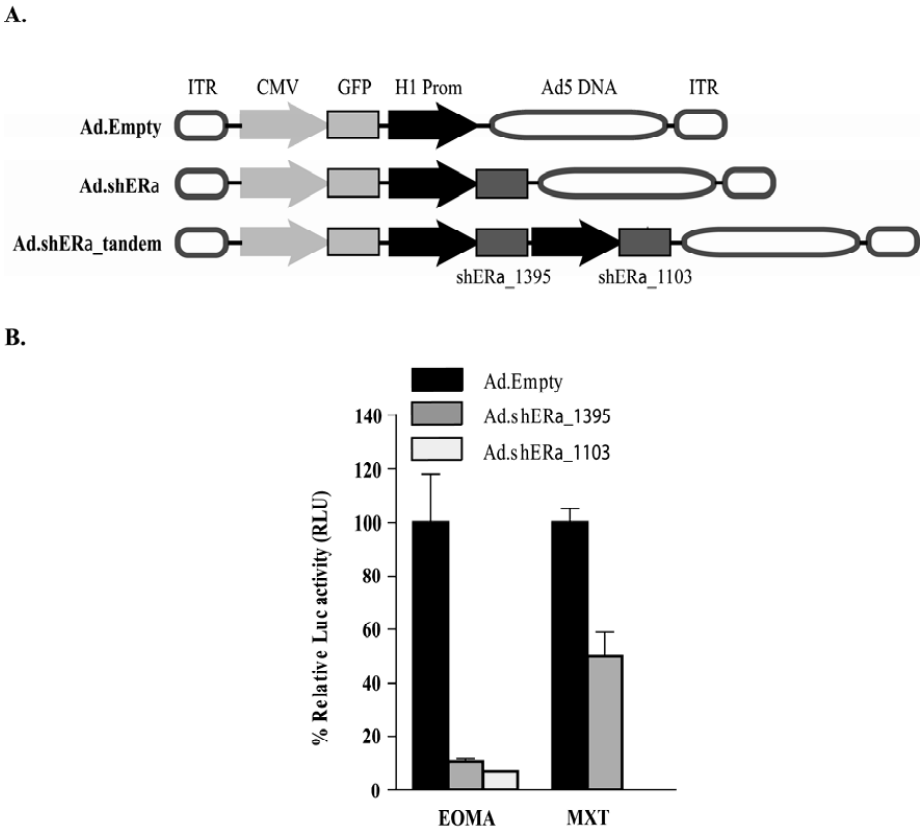


Figure 2. ER-mediated luciferase activity after Ad-mediated transfer of shERα *in vitro*
(A) Schematic representation of the recombinant Ad vectors, carrying GFP and shERα expression cassettes that were used in this study. (B) EOMA and MXT cells were co-transfected with pERE-Luc and pCMV-LacZ and than infected either with Ad.Empty, Ad.shERα_1395, or Ad.shERα_1103. 10^{-9} M Estrogen was administrated for 24 hours. Luciferase activity was measured 48 hours after infection. Data represented as mean ± SD relative to infection with Ad.Empty.

We then proceeded with the application of our vectors *in vivo*. The Ad vectors (Ad.Empty, Ad.shERα_1395, or Ad.shERα_tandem) were injected in the tail vain of C57Bl/6 mice. This allowed examination of inhibition by shERα of endogenous hepatic mERα. Four days post-injection, animals were sacrificed, and the livers were studied for GFP expression and ERα mRNA level. Similar GFP expression patterns were observed in all groups, indicating equally efficient transduction (data not shown). ERα mRNA levels were studied by

real time PCR analysis (Fig. 3). Administration of Ad.shERα_1395 reduced ERα mRNA levels 70%, whereas hepatic expression of shERα_tandem resulted in an 85% reduction.

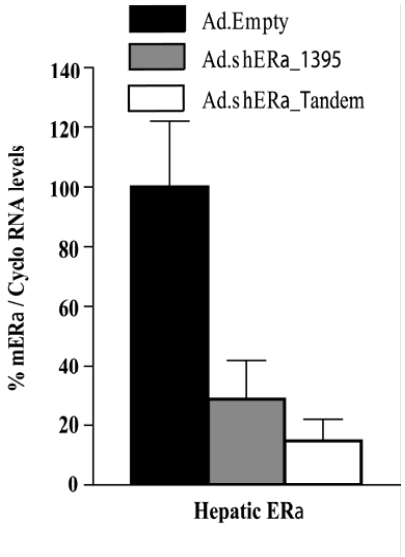
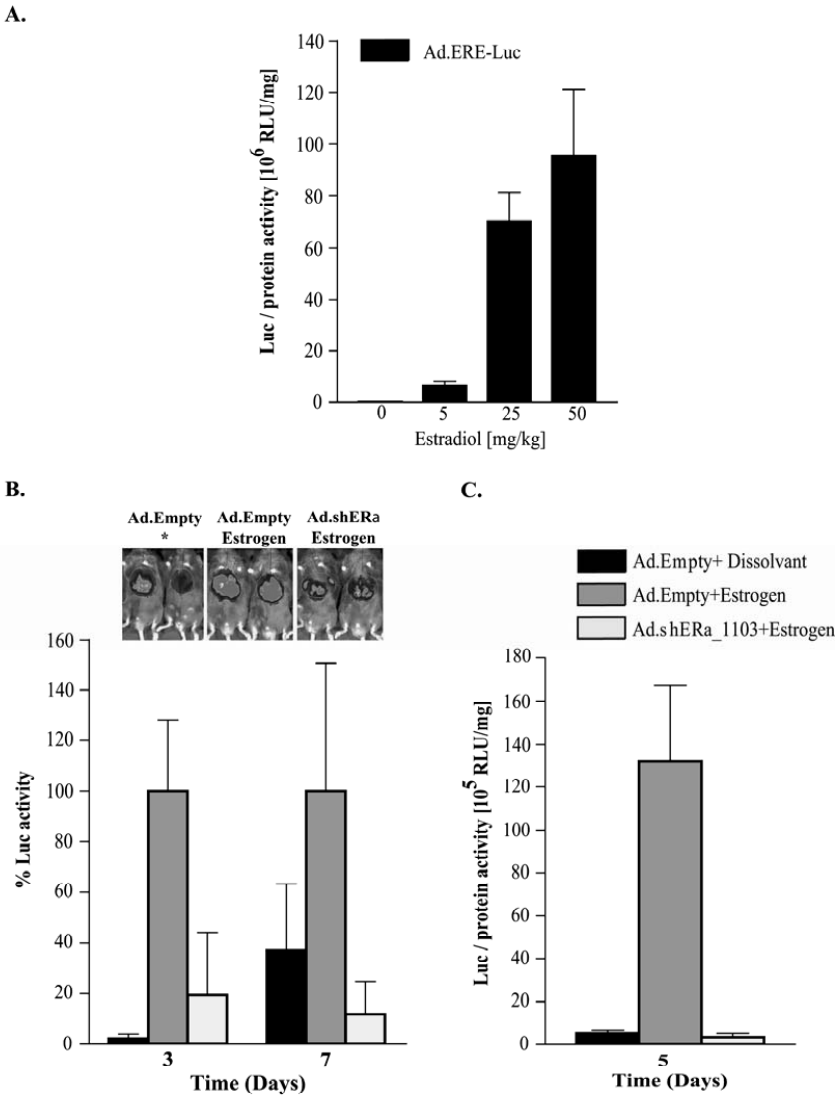


Figure 3. Hepatic ERα mRNA levels after Ad-mediated transfer of shERα *in vivo*. Male C57Bl/6 mice (n=5) were injected with $4x10^9$ pfu Ad.Empty, Ad.shERα_1395 or Ad.shERα_tandem. Livers were harvested four days after Ad. administration and subjected to taqman analysis. The cyclophillin gene was used as internal standard. Data represented as mean ± SD.

Subsequently, we sought to examine the extent of shERα-mediated repression of hepatic mERα transcription activity. For this purpose, we constructed an Ad vector carrying the estrogen responsive luciferase reporter gene (Ad.ERE-Luc). First the estrogen-responsiveness of this vector was determined *in vivo* (Fig. 4A). Five days post-injection of $8x10^8$ pfu Ad.ERE-Luc, the mice were injected s.c with increasing concentrations of estrogen, ranging from 0 to 50 μg/kg. As shown in Fig 4A, six hours post-injection, estrogen induced hepatic luciferase activity in a dose-dependent manner. Maximal stimulation was reached after applying 25 μg/kg estrogen. Then, we determined to what extend Ad.shERα down-regulates the transcriptional activity of hepatic ERα. Ad.shERα together with Ad.ERE-Luc reporter vector was administrated intravenously to C57BI/6 mice. Luciferase expression was detected by a CCD camera in living mice. Without estrogen treatment, all mice exhibited the same basal expression of the reporter construct (data not shown). Administration of 5 μg/kg estrogen, three and seven days after transduction with Ad.shERα_1103, resulted in a significant repression of hepatic ERα-mediated luciferase activity (Fig. 4B). These data were confirmed by measuring luciferase activity in liver extracts of mice that received estrogen (5 μg/kg, sc) five days post-injection with Ad.ERE-Luc plus Ad.Empty or Ad.shERα_1103 (Fig. 4C).

We conclude that Ad-mediated introduction of shERα *in vivo* results in an almost complete repression of hepatic mERα mRNA levels, as well as mERα-mediated transcription activity.



[8] pfu Ad.LacZ, were injected with $8x10^8$ pfu Ad.ERELuc. Five days later, the recipients were treated for 6 hours with increasing amounts of estrogen (0-50 µg/kg, s.c). Then, the mice were sacrificed, and the livers were processed for luciferase assays. Luciferase activity is expressed as relative luciferase units (RLU) per mg total liver protein. (B) Male C57Bl/6 mice (n=5) were injected with Ad.ERE-Luc ($5x10^8$ pfu) plus Ad.Empty or Ad.shERα_1103 ($3x10^9$ pfu). Three or seven days post-infection, the mice were injected with 5 µg/kg estrogen. The (inset) photo shows the result of optical imaging of the bioluminescence at day three, the bar-diagram is a quantitative representation of hepatic luciferase activity at day three or day seven. (C) Male C57Bl/6 mice (n=5) were co-injected with Ad.ERELuc ($5x10^8$ pfu) + Ad.Empty or Ad.shERα_1103 ($3x10^9$ pfu). Five days later, the mice received 0 or 5 µg/kg estrogen. After 6 hours, the animals were sacrificed, and hepatic luciferase activity was determined. Luciferase activity is expressed as relative luciferase units (RLU) per mg total liver protein. Data represented as mean ± SD.

Discussion

In this paper, we demonstrate that efficient silencing of mouse ERα can be achieved *in vitro* as well as *in vivo* by use of Ad-mediated transfer of shRNA molecules that target the ERα mRNA. Two independent shERα plasmid and Ad vector expression constructs were generated and shown to be effective in repressing endogenous ERα activity up to 80% in several different cell lines and *in vivo* (Fig. 1A, 2B and 3). In addition, a construct was made expressing both shERα sequences simultaneously. *In vitro* as well as *in vivo,* this construct was shown to be more effective (Fig. 1A and 3) than either of the two shERα constructs alone. Non-invasive optical imaging of living mice, allowed us to quantify shERα activity *in vivo.* Significant reduction of mouse ERα transcription levels were observed up to seven days post-transduction (Fig. 4B).

Thus far, bystander effects caused by shRNA constructs targeted to an unrelated gene have not been reported, and the specificity of the shERα_1395 construct was verified by the observation that human ERα, which has a single mismatch with the murine ERα target sequence, is not down-regulated (Fig. 1C). The number of mismatches with the murine ERβ sequence totals five, making it unlikely that the shERα_1395 construct would affect expression of ERβ. Similarly, the shERα_1103 construct has three mismatches with the human ERα and nine mismatches with murine ERβ, making it unlikely that the shERα_1103 construct would interfere with either of them. A single mismatch in the shERα_1395 sequence did render the construct ineffective in down-regulating murine ERα (data not shown). Thus, the two independent shERα constructs described here are exquisitely suited to demonstrate that a specific effect is mediated by down-regulation of ERα expression and not by down-regulation of a related sequence.

A key challenge in the application of an shRNA based approach is efficient delivery of the shRNA constructs to target cells *in vitro* and *in vivo*. For application of shRNA *in vivo*, the sh oligopair, driven by H1 RNA polymerase [2] or U6 promoter [6], can be cloned in viral vectors. Here, the Ad vector was chosen as delivery vector, because of the relative ease of generation and amplification. Moreover, the natural tropism of Ad vectors for the liver enables the rapid analysis of the hepatic knock-down phenotype. Since Ad vectors predominantly infect the parenchymal cells [7,8], it is important to note that most abundant hepatic ERα expression was detected in parenchymal cells while ERα expression was barely detected in hepatic endothelial cells or kupffer cells (data not shown). This supported the rationale for application of shERα Ad vectors *in vivo*. Another interesting observation was

that upon administration of $4x10^9$ pfu Ad.shERα, an 85% reduction of ERα mRNA levels was obtained (Fig. 5), whereas co-injection of $3x10^9$ pfu Ad.shERα_1103 with $5x10^8$ pfu Ad.ERE-Luc resulted in an almost complete absence of luciferase activity (Fig. 4C). The ratio of Ad.ERE-Luc *versus* Ad.shERα_1103 (1:6) should ensure that all cells that were transduced by Ad.ERE-Luc also received Ad.shERα_1103. Thus, the remainder of ERα expression determined by real-time PCR likely reflects ERα expression in non-parenchymal and non-infected cells.

Thus far, relative few reports describe the application of Ad vectors as delivery system for RNAi *in vitro* [9-12]. Similarly, relative few studies on effective RNA interference *in vivo* using Ad mediated gene transfer have been reported [13-15]. One potential explanation for this relative paucity in the application of Ad mediated gene transfer for shRNA expression constructs could lie in the recent observations of Lu and Cullen [16]**,** that VA1 non-coding RNA, expressed by wild type adenovirus is a potent inhibitor of RNA interference. However, replication-incompetent adenovirus vectors such as the vectors used in our study have been reported to express low levels of VA1. Moreover, in our hands the effect of the pSUPER shRNA construct shERα_1935 on reduction of ERα activity in vitro was not affected by super-infection with the Ad.empty vector (data not shown). Thus, the Ad vectors applied in this study seem to have no or a minor inhibitory effect on the RNAi response in vitro and in vivo. Whether this effect is also insert specific and/or depends on the particular target gene remains to be investigated.

The strongest evidence for efficient reduction of endogenous hepatic ERα RNA levels *in vivo* was obtained by co-injection of Ad.ERE-luc and advanced non-invasive *in vivo* optical imaging. Administration of Ad.ERE-luc led to readily detectable levels of luciferase activity from day 3 up to day 7 and disappeared at day 10 (data not shown). In agreement with this, the Ad.shERa mediated knock-down effect was present at day three, five, and seven post-injection (Fig 4B). This represents a 4 to 5-day window of expression to determine the phenotypic effects of hepatic shRNA-mediated reduction of mRNA levels.

Conclusion

We have shown significant repression of hepatic ERα activity in mice utilizing Ad.shERα vectors. In addition, using advanced non-invasive optical imaging technology, the dynamics of the knock-down effect *in vivo* have been demonstrated. Thus, our data confirm that application of shRNA represents a powerful tool for targeted gene silencing. We conclude

that Ad-mediated delivery of shERα constructs represents an elegant tool to gain more insight in the role of the hepatic ERα.

Methods

Plasmids

Two oligonucleotide pairs (mERα_1395: 5'-gatcccc**gctcctgtttgctcctaac**ttcaag agagttaggagcaaacaggagctttttggaaa-3' and 5'-agctttttccaaaaagctcctgtttgctcctaa ctctcttgaagttaggagcaaacaggagcggg-3', mERα_1103: 5'-gatcccc**gaatagccctgc cttgtcc** ttcaagagaggacaaggcagggctattc tttttggaaa-3' and 5'-agc ttttccaaaaaga atagccctgccttgtcctctcttgaaggacaaggcagggctattcggg) were ordered (Eurogentec, United kingdom). The bold nucleotides correspond to nucleotides 1395-1418 and 1103-1120 of the mRNA mERα sequence (GenBank accession number NM_ 007956). The underlined nucleotides represent a *Bgl*II and a *Hind*III site. These oligo's were annealed and ligated between the *Bgl*II and *Hind*III sites of pSUPER-H1prom [2]. The pSUPER-shERα sequences were verified by restriction and sequence analysis (ABI 3700, LGTC, Leiden).

The H1prom plus or minus shERα were cloned from the pSUPER into the promoter less pAdTrack vector [5] by use of *Xba*I and *Xho*I restriction sites. The Ad.shERα_tandem construct was generated by ligation of H1prom-shERα_1103 between the *Not*I and *Kpn*I sites of pTrack-H1prom- shERα_1395.

The $(ERE)_3$TATA-Luc was cloned from pGl_3-basic as a *Cla*I-blunt/ *Kpn*I fragment in *EcoR*V- and *Kpn*I- digested promoter less Shuttle vector (pShuttle) (He et al. 2509-14)**.** The functionality of this construct was verified by transfection. hERα was cloned from pCMV5 (pCMV5-hERα) [17] as a *BamHI* fragment in the *BglII* digested pShuttle-CMV vector. The pcDNA3.1-mERα expression vector was provided by Larry Jameson [18] and subcloned as a *EcoRI*-blunt fragment in the *EcoRV* digested pShuttle-CMV vector.

Cell Culture

The MXT^+ (murine breast cancer) cell line was generously provided by Dr. Bernards. H5V (a murine endothelial cell line derived from heart), EOMA (murine hemangioma-derived micro vascular cell line) and MXT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum, 100 units/ml Penicillin, 100 μg/ml Streptomycin and glutamax (Invitrogen) (Complete DMEM). PERC6 cells [19] were maintained in complete DMEM supplemented with 10mM $MgCl^{2+}$.

For large-scale production of recombinant Ad in PERC6 cells (Crucell, Leiden, he Netherlands), complete DMEM with 2% horse serum (Gibco) was used.

Luciferase reporter assays

Transient transfections were performed in triplicate in 12-wells plates (1.10^5 cells per well) using Lipofectamine (Invitrogen). The effect of shERα on ERα mediated transcription regulation was determined by co-transfecting the cells with 100ng of reporter construct $(ERE)_3$TATA-LUC and 500 ng expression vector pSUPER-shERα or an empty pSUPER control vector together with 100 ng pCMV-LacZ. After 24 hours, the cells were stimulated with complete DMEM containing 10^{-9}M Estrogen for an additional 24 hours. The cells were lysed with reporter lyses buffer (Promega) and after centrifugation of 2 min, supernatant was used for determining β-galactosidase normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of cell extract in a monolight luminometer (BD Biosciences). β-galactosidase was measured in a 96-well microtiter plate using the β-Galactosidase Enzyme Assay System in reporter lyses buffer (Promega). Absorbance at 450 nm was determined in a microplate reader. Luciferase activities were normalized for transfection efficiency with the β-galactosidase activity and expressed as a percentage relative to expression levels induced by endogenous estrogen receptor (ER). Expression of endogenous ERα in those cells was verified by real time PCR.

Western blot analysis

Immunoblotting procedures were as described previously [20]. H5V cells seeded in triplicate in 12-wells plate were co-transfected with 20 ng pCMV-mERα and 500ng expression vector pSUPER-shERα or an empty pSUPER control vector as described above. 28 hours post-transfection, the cells were lysed in 200 µl of RIPA buffer (1% NP40, 0.5% DOC, 0.1% SDS, 50mM Tris pH 8.0, 150mM NaCl, 2.5mM EDTA) containing protease inhibitor (40ul/ml, Roche). Extracts were cleared by centrifugation (4°C, 14 000 *g*, 5 min), and protein content was determined using the BCA kit (Pierce). Protein samples were denaturated (5 min, 90°C) and separated on SDS/PAGE by use of 8% gradient gels and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). Blots were stained with Ponceau S before blocking to verify equal loading and appropriate protein transfer. Membranes were blocked for 90 min in PBS, pH 7.4, containing 0.05% Tween 20 and 10% milk powder. Thereafter, membranes were incubated for 16 h at 4°C with ab MC20, 1:1000 (mERα rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). After extensive washing with blocking buffer without milk powder or BSA, membranes were

incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000 (Promega). Membranes were again extensively washed and bound peroxidase conjugates were visualized by enhanced chemiluminescence (ECL, Amersham) on a LumiImager workstation. Additionally, filters were stripped by a 30 min incubation in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.8 at 50°C, to proceed with the whole procedure as described above. However, now membranes were incubated for 16 h at 4°C with p-38 ab, 1:1000 (N-20, cs-728, rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). Immunoblots were quantified using LUMIANALYST software on a LumiImager (Boehringer-Mannheim).

Adenoviral vectors

Recombinant adenoviral plasmids were generated by homologous recombination of pAdtrack or pShuttle vectors with pAdEasy1 in BJ5183 cells as described previously [5]. Correct clones were propagated in DH5α cells (Life Technologies). For the generation of the Ad.shERα vectors, Ad.Empty and Ad.ERE-Luc, PERC6 cells were transfected with 4 μg Pac-I-linearized adenoviral construct using LipofectAMINE PLUS (Life Technologies). After 16 hours transfection medium was replaced by growth medium. Transfected cells were harvested at day seven post-transfection and after three freeze-thaw cycles the lysate was used for large-scale production of Ad vectors in PERC6 cells. Virus was purified by double CsCl centrifugation and subsequently dialysed as described previously [21]. Final yields as assessed by plaque assays on 911 cells were approximately 2×10^{10} plaque forming units (pfu)/ml. The control virus (Ad.Empty) carries the green fluorescent protein (GFP) under control of cytomegalovirus promoter (CMV) and contained the H1prom. Ad.shERα_1395 and Ad.shERα_1103 carry GFP under control of CMV and shERα_1395 or shERα_1103 under control of H1prom. Ad. shERα_tandem carries both shERα_1395 and shERα_1103 under control of their own H1prom. Ad.ERE-Luc does not contain CMV-GFP and its functionality was verified *in vitro* and *in vivo.*

Infection cells

24 hours before transfection, 1.10^5 cells per well were seeded into12 wells-plate. Cells were transiently transfected by use of lipofectamine with a total of 450ng of DNA per well (150ng of reporter plasmid $(ERE)_3$TATA-LUC and 300ng pCMV-LacZ). After 4 hours cells were infected with either Ad.shERα or control Ad.Empty (MOI 5.000). Additionally, they received 10^{-9}M estrogen for 24 hours. Cells were lysed in 300 μl reporter lyses buffer. β-galactosidase and luciferase activity was determined as described above.

Animals and Ad Injection

The Ethics Committee for Animal Experiments of the Leiden University approved all animal work and the experimental protocols complied with the national guidelines for use of experimental animals. Male C57Bl/6JIco (Charles river, The Netherlands) were given a standard m diet Chow (Hope Farms, Woerden, NL) and housed under standard conditions in conventional cages with free access to water and food.

Recombinant Ad, with a maximum of 4×10^9 pfu in 200 µl of PBS, were administered by injection into the tail vein of mice at the age of 14 weeks. Within five days post-infusion, mice were sacrificed; liver pieces were removed and immediately deep-frozen in liquid nitrogen and stored at -80°C.

Pharmacological treatment.

The experiment was carried out in 12-wks old C57BL/6 male mice. To prevent sequestration of low doses of Ad.ERE-Luc by liver Kupffer cells, mice were pre-injected with Ad.LacZ ($5x10^8$ pfu) 4 hours before administration of $8x10^8$ pfu Ad.ERE-Luc. 17β-estradiol (Sigma, E8875) was dissolved in sesame oil (Sigma). In the dose-response experiment, five days post-injection of Ad.ERE-Luc, 0, 5, 25 and 50 µg/kg 17β-estradiol was injected for 6 hours. Then liver pieces were rapidly dissected and immediately deep-frozen in liquid nitrogen and stored at -80°C for further analysis.

Bioluminescent reporter imaging.

The experiment was carried out in 12 wks old C57BL/6 male mice co-injected with Ad.ERELuc ($5x10^8$ pfu) plus either Ad.Empty or Ad.shERα ($3x10^9$ pfu). Bioluminescent signals (BLS) were performed at time 0 and at several days after 6 and 24 hours s.c injections of 5 µg/kg 17 β-estradiol with the Xenogen IVIS imaging system (IVIS 100). The living mice were intraperitoneal (ip) injected with the luciferase substrate, luciferin, at a dose of 150 mg/kg body weight approximately 5 minutes before imaging. The mice were anaesthetized with isoflurane/oxygen and placed on the imaging stage. Total photon emission of each animal was acquired for 1 minute. Captured images were then quantified by using the Living Image software (Xenogen Corp, Almeda, CA) and the IGOR software (WaveMetrics Corp, Lake Oswego, OR). BLS from the region of interest (ROI) was expressed using the pseudo colour scale (Red most intense and Blue least intense luminescence) and the data were presented as the cumulative photon counts collected within each ROI. Because layers of tissue may limit photon emission from inner organs, the experiment was repeated. Of these mice the livers were rapidly dissected at day 5, 6 hours after 17β-estradiol administration, verifying the

results from the bioluminescent reporter imaging by determining the luciferase activity in liver lysates

Luciferase enzymatic assay.

The liver extracts were prepared by homogenisation with the minibead beater in reporter lyses buffer (Promega), two cycles of freeze-thawing and 2 min. of centrifugation at maximum speed. Supernatants were used for determining protein-normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of liver extract in a monolight luminometer (BD Biosciences). Protein content was measured in a 96-well microtiter plate using the BCA protein assay kit (Pierce). Absorbance at 562 nm was determined in a microplate reader.

Real time quantitative PCR analysis

Total RNA was extracted from liver using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [22]. PCR primer sets (Cyclophilline, Fw: AAAAGGAAGACGACGGAGCC Rev: TCGGAGCGCAATATGAAGGT and mERα, Fw: CTAGCAGATAGGGAGCTGGTTCA, Rev: GGAGATTCAAGTCCCCAAAGC) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. Cyclophilline was used as a control.

Data Analysis—The significance of differences in relative gene expression numbers C_t ($C_{t((Cyclo)}$–$C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a two-tailed Student's *t* test. Probability values less than 0.05 were considered significant.

Authors' contribution

Y.K carried out the studies described in this paper and drafted the manuscript. F.F participated in the concept of designing shERα. I.Q and C.L. contributed to the imaging experiments shown in Fig 4B. K.WvD participated in the design and coordination of this study and provided expert input for writing the manuscript. All authors read and approved the final manuscript.

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3.

Repression of Hepatic Estrogen Receptor Alpha Does Affect Expression of Lipid-Related Gene but Does Not Affect Lipid Metabolism in Female APOE*3 Leiden Mice.

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Abstract

Estrogens have been shown to modulate the lipoprotein profile. However, the role of the hepatic estrogen receptor α (ERα) in this process is unclear. In the present study, we have addressed the role of hepatic ERα signalling in lipid metabolism of APOE*3 Leiden transgenic mice fed a high fat diet. Hepatic ERα was down regulated using adenovirus-mediated transfer of a short hairpin (sh) RNA directed against the ERα (Ad.shERα). Despite significant down-regulation of hepatic ERα RNA and protein levels (60%), plasma cholesterol, triglyceride and glucose levels were not changed. In addition, no effects on the VLDL-TG secretion rate and intra-hepatic lipid levels were observed. In contrast, expression of the Cyp7a and PPARα genes was up regulated 2- and 2.5-fold, respectively, and the SHP gene was down regulated 2-fold. Apparently, the changes in the expression of these lipid related genes is compensated for by alternative transcriptional or post-transcriptional mechanisms and does not affect plasma lipid levels. In conclusion, repression of hepatic ERα gene expression does affect genes involved in lipid metabolism, but does not have an obvious impact on lipid parameters.

Introduction

Epidemiological studies have shown that the menopausal transition is association with changes in circulating lipid levels, including elevated plasma levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), and reduced levels of high-density lipoprotein cholesterol (HDL-C). Since estrogen treatment has been reported to influence these lipid levels in the opposite manner [1-4], estrogen has been postulated to be beneficial in cholesterol homeostasis.

The estrogenic effects are predominantly mediated via activation of either of two estrogen receptors (ERs), ERα and ERβ. These ligand-dependent transcription factors modulate gene transcription but can also interfere with intracellular signaling pathways [5-7]. To date, mouse models of estrogen deficiency, such as aromatase knockout (ArKO) and ERα and ERβ knockout mice have been used to gain insight into the role of estrogens in lipid metabolism. ArKO mice, ERα - and double ERα/β knockout mice all develop hypercholesterolemia [8-10], whereas no lipid phenotype was described in ERβ knockout mice [10]. These results demonstrate a role for estrogen in lipid homeostasis, and indicate that ERα is involved.

The liver plays a central regulating role in lipid metabolism. To gain insight into the role of hepatic ERα in lipid homeostasis, Ad vectors encoding shRNA's directed against mouse ERα (Ad.shERα) [11] were administered to hyperlipidemic APOE*3-Leiden female mice. Hepatic ERα mRNA and protein levels were repressed by 60%, and were associated with changes in the expression level of genes involved in lipid metabolism. However, plasma lipid parameters were not affected upon Ad.shERα administration. These results indicate that the hepatic ERα level does not play a rate limiting role in lipid metabolism.

Results

Basal body weight and plasma parameters

To induce hyperlipidemia, two groups of female APOE*3-Leiden mice were fed a high fat cholesterol enriched diet (diet W) for eight weeks. After this period, mice in both groups had an average bodyweight of 21 gram, serum glucose levels of 6 mM and triglyceride (TG) level of 1.9 mM. In addition, both groups of mice exhibited hypercholesterolemia (13.4 and 13.8 mM) (table 1).

Table 1. Bodyweight and glucose levels in 4 hrs-fasted ApoE*3-Leiden female mice fed a high fat diet, before and after Ad-mediated gene transfer of shERα

	Day 0		Day 5	
	Ad.Empty	**Ad.shERα**	**Ad.Empty**	**Ad.shERα**
Bodyweight (gr)	20.9 ± 1.4	21.2 ± 1.2	20.1 ± 1.2	20.2 ± 1.3
Glucose (mmol/l)	6.1 ± 1.0	5.8 ± 1.1	6.5 ± 0.7	6.6 ± 0.8
Cholesterol	13.4 ± 4.4	13.8 ± 3.3	8.6 ± 0.9	8.6 ± 1.4
Triglycerides	1.9 ± 0.9	1.9 ± 0.5	3.2 ± 0.5	3.1 ± 0.6

Hepatic ERα levels in ApoE*3-Leiden mice after Ad.shERα treatment

To down-regulate the hepatic ERα, the hyperlipidemic female APOE*3-Leiden mice were injected either with Ad.Empty or with Ad.shERα. Five days after Ad.shERα treatment ($1.5.10^9$ pfu), the hepatic ERα RNA and protein levels were repressed by 60% as compared to Ad.Empty treated mice ($P<0.05$, $P<0.001$, respectively; Figure 1A and 1B).

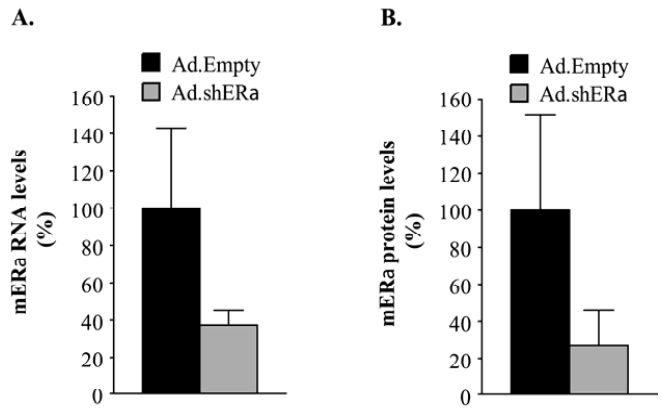

Figure 1. Hepatic ERα levels after Ad mediated transfer of shERα *in vivo*
Female APOE*3-Leiden mice were injected with $1{,}5.10^9$ pfu Ad.Empty or Ad.shERα (n=5). Livers were harvested five days after Ad. administration and subjected to taqman (A) and western (B) analysis. Respectively, HPRT and p38 were used as internal standard. Data represent as mean ± SD

Body weight and plasma parameters in Ad.shERα treated mice

At day five after Ad administration, cholesterol and TG levels were modulated to the same extent in both Ad.Empty and Ad.shERα treated mice (Table 2). Thus, down-regulation of hepatic ERα did not affect serum lipid nor glucose levels.

Hepatic VLDL-TG production

After down regulation of hepatic ERα in APOE*3-Leiden mice, the VLDL-TG production rate was determined by injection of Triton WR1339. Triton WR1339 blocks VLDL-TG lipolysis and VLDL remnant clearance and the increase in plasma TG is a measure for VLDL-TG production. Reduced hepatic ERα levels did not affect the VLDL-TG production rate (Figure 2).

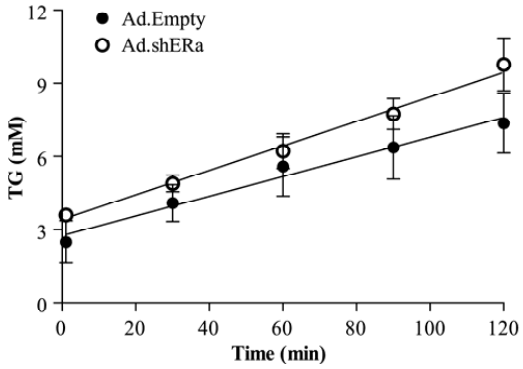

Figure 2. Hepatic VLDL-TG production after Ad mediated transfer of shERα *in vivo*
Female APOE*3-Leiden mice were injected with $1{,}5.10^9$ pfu Ad.Empty or Ad.shERα (n=4). VLDL-TG production was measured 5 days post-injection. Fasted serum TG level was determined between 0 and 120 min after Triton WR 1339 injection. Values are represented as mean ± SD.

Hepatic Lipid content

In addition, hepatic TG and Chol content were analyzed. As depicted in Figure 3, the hepatic lipid content of the Ad.shERα treated APOE*3-Leiden mice did not differ from the Ad.Empty treated group (Chol; 14.4 ± 1.7 versus 13.8 ± 2.9, for TG; 104.5 ± 38.7 versus 93.1 ± 31.9, for Chol esters; 33.8 ± 7.6 versus 29.9 ± 6.2 mM, respectively).

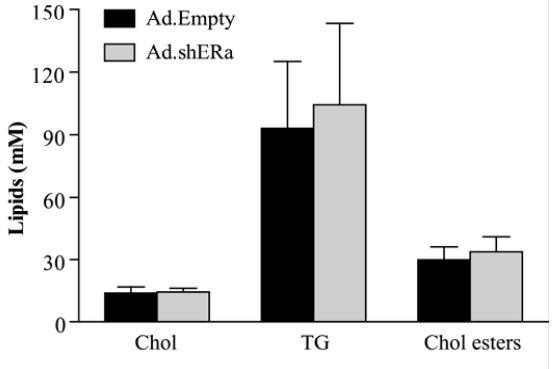

Figure 3. Hepatic lipid content after Ad mediated transfer of shERα *in vivo*
Hepatic TG and cholesterol content was analyzed in APOE*3-Leiden female mice five days after Ad.Empty and Ad.shERα administration. Values represent the mean±SD of 11 mice.

Hepatic mRNA expression levels

To further investigate the effect of short-term repression of ERα in liver, hepatic expression of genes involved in lipogenesis were assessed by real-time PCR. Short-term repression of hepatic ERα led to a significant enhancement of peroxisome proliferator-activated receptor

(PPAR)α and Cyp7α and a significant repression of short heterodimer partner SHP (Fig 4). Transcription levels of apolipoprotein E (ApoE) and ApoAV were unchanged (Fig 4).

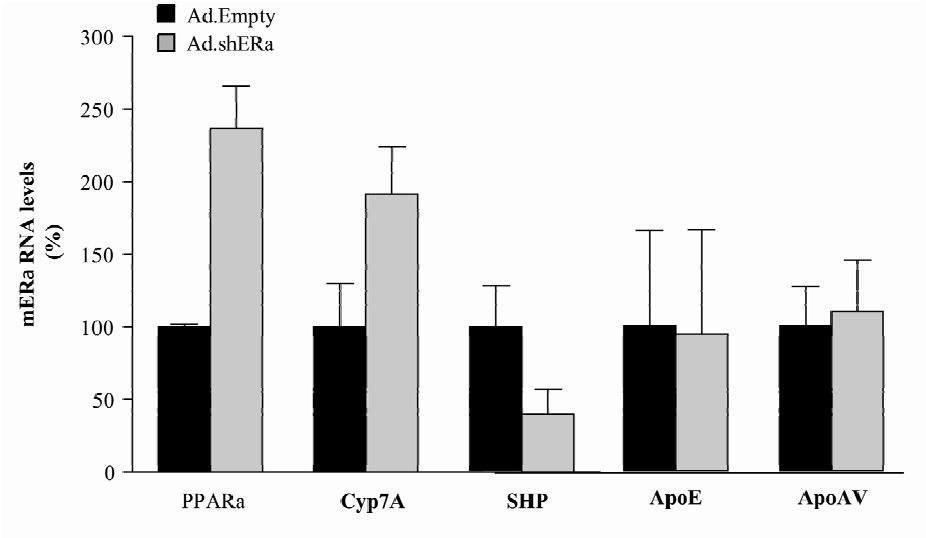

Figure 4. Hepatic gene expression after Ad mediated transfer of shERα *in vivo*
Gene expression was analyzed by real time PCR in APOE*3-Leiden female mice five days after Ad.Empty and Ad.shERα administration. The HPRT gene was used as internal standard. Values represent the mean±SD (n=5) relative to the percentage of expression in Ad.Empty treated mice. *, statistically significant difference of $P<0.05$ compared with Ad.Empty treated mice.

Discussion

The present study evaluates the direct role of hepatic ERα in lipid homeostasis. To this end, hepatic ERα was down-regulated in hyperlipidemic APOE*3-Leiden female mice using Ad mediated transfer of a shRNA construct targeted against the ERα. This resulted in a 60% reduction in hepatic ERα RNA and protein levels and significant changes in PPARα, Cyp7α and SHP gene transcription. However, hepatic lipid levels and serum lipid and glucose levels were not affected by ERα down-regulation. Apparently, the hepatic ERα is involved in regulating hepatic gene transcription, but ERα level does not play a rate limiting role in determining serum lipid or glucose levels in hyperlipidemic APOE*3Leiden mice.

The application of vector-based systems expressing small hairpin RNA (shRNA) to dissect gene function is now well established in mammalian cells in vitro. In vivo application requires highly efficient delivery of the shRNA expression construct and for this, in the current paper Ad vectors are used. These have been shown to efficiently knock-down ERα in

liver in vivo, resulting in highly efficient reduction of ERα transcriptional activity [11]. Ad vectors predominantly transduce hepatic parenchymal cells, which also most abundantly express ERα (data not shown) [12,13]. We were able to repress hepatic ERα RNA and protein levels to an extent of 60% with a moderate viral dose ($1,5.10^9$ pfu/mice). At higher viral dosages, the reduction in gene expression was not further increased and hepatotoxicity did occur (data not shown).

We previously demonstrated that the shERα construct used here is specific for the murine ERα [11]. Since the ERβ is expressed at very low levels in parenchymal cells, and our shRNA construct harbours nine mismatches with the murine ERβ, it seems unlikely that off target effects explain the effect of shERa expression on hepatic gene expression. However, we cannot exclude that some of the effects on gene expression are mediated by the so called a-specific interferon response to double stranded RNA. However, it is likely that the Ad transduction per se induces a much more dramatic cellular stress response as compared to the dsRNA. This ad specific effect was controlled for in the comparison with the ad empty treated groups.

The reduction in ERα level is associated with significant changes in the expression of genes involved in lipid metabolism. The Cyp7α gene encodes the enzyme controlling the first and rate-limiting step in cholesterol degradation to bile acids. Upregulation of the Cyp7α gene is in line with the observed down regulation of SHP, as SHP negatively regulates expression of Cyp7α. Interestingly, SHP appears to be induced by estrogen in liver of wt mice [14,15], and a decrease of estrogen signaling would thus be in line with SHP downregulation. Agonists of the transcription factor PPARα prevent lipid accumulation in liver by stimulating fatty acid β-oxidation in liver [16,17] a process which is also found to be induced by estrogens [18]. The upregulation of PPARa in response to a reduction in Era could therefore be a compensatory effect. Since we did not find a lipid phenotype associated with these gene expression changes, it seems likely that the gene expression changes them selves or post-transcriptional regulatory events counterbalance each other. Apparently, the liver can compensate for changes in estrogen signaling in such a manner as to maintain normal plasma and liver lipid levels.

The role of ERα in lipid metabolism has been addressed using whole-body knockout mouse models lacking ERα. These ERα knockout mouse models as well as the aromatase knock out mouse model, which lacks the final step in estrogen synthesis, display a lipid phenotype that is apparent upon aging [10,19,20]. The phenotype associated with any

knockout mouse model needs to be considered with the provision that compensatory changes that counteract some of the knockout effects may have occurred. The delayed lipid phenotype of the ERα knockout and ARKO mouse models could be explained by a failure of this compensation in time. By reducing the hepatic ERα mediated signalling cascade during adulthood and assessing the parameters relatively soon thereafter, our data indicate that the lack of a lipid phenotype of ERα knock outs is not due to compensatory changes. In stead, we conclude that hepatic ERα level is not rate-limiting in its role to maintain whole body lipid metabolism.

If the liver is not directly mediating the effects of estrogen on lipid metabolism, what could be the cause of the lipid changes seen after prolonged absence of the ERα or estrogen? Although we have not addressed this, it seems likely that secondary effects of estrogen signaling on other tissues that are involved in regulating lipid metabolism, such as brain, muscle and adipose tissue, play an important role in the development towards a change in lipid profile upon aging. In this respect, changes in for example adipose tissue distribution and size, as have been attributed to estrogen, would only have an effect on lipid metabolism beyond a certain level of change and thus time.

In conclusion, we find that short-term repression of hepatic ERα gene and protein expression does not have an overt impact on plasma and liver lipid levels. Apparently, the changes induced via the hepatic ERα are effectively compensated for or play a relatively minor role in maintaining cholesterol and triglyceride homeostasis.

Methods

Plasmids and Adenoviral vectors

The p.Empty, p.shERα plasmids and the Ad.Empty, Ad.ERE-Luc and Ad.shERα vectors have been generated as previously described [11].

Animals and Ad Injection

All animal work was approved by the Animal Ethic Committee from the Leiden University Medical Center and TNO-Prevention and Health, Leiden, the Netherlands and the experimental protocols complied with the national guidelines for use of experimental animals. APOE*3-Leiden female mice were housed under standard conditions in conventional cages with free access to water and food. The study was performed in 17-19 weeks old APOE*3-Leiden mice (n=11) that were fed a Western type diet (Hope Farms, Woerden, The

Netherlands) starting 8 weeks prior to the experiment. For Ad-mediated gene transfer experiments, mice were transferred to filter-top cages, placed in a designated room, and allowed to adapt for at least five days. For in vivo adenoviral transductions, $2x10^9$ plaque forming units Ad.shERα or Ad.Empty in total volume of 200 µl (phosphate-buffered saline) were injected into the tail vein of mice. Within five days post-infusion, mice were sacrificed; liver pieces were removed and immediately deep-frozen in liquid nitrogen and stored at -80°C.

Plasma parameters

At day –6 and day 5 of Ad. injections, APOE*3-Leiden female mice were fasted for 4 h. Blood samples were taken via tail bleeding in paraoxon-coated capillaries, to prevent lipolysis [21]. Plasma was collected by centrifugation at 4°C. Plasma levels of total Chol and TG were determined enzymatically using commercially available kits and standards (Sigma Diagnostics, St. Louis, MO; Roche Molecular Biochemicals GmbH, Mannheim, Germany; and Wako Chemicals GmbH, Neuss, Germany). Blood glucose levels were measured by a Freestyle hand glucose analyzer (Disetronic, Vianen, The Netherlands). All plasma parameters were determined according to the manufacturers' instructions.

Hepatic VLDL-TG production

At day 5 after $1.5.10^9$ pfu Ad.Empty or Ad.shERα administration, APOE*3-Leiden female mice were fasted for 4 h and then intravenously injected with 500 mg/kg Triton WR 1339 (Sigma) as described [22]. Blood samples of Ad.Empty and Ad.shERα treated mice were collected 1, 30, 60, 90 and 120 min after Triton injection (n=4 and n=5 respectively). Serum TG concentrations were measured enzymatically, as described above. The hepatic VLDL-TG production rate was measured as the accumulation of serum TG after Triton injection and expressed as mg/dl/min.

Hepatic Lipid levels

Liver and muscle samples were homogenized in H_2O (~10% wet wt/vol). Lipids were extracted according to Blight and Dyer's method [23]. In short, a solution was made of each sample of 200 µg protein in 800 µl H_2O. 3 ml methanol/chloroform (2:1) was added and mixed thoroughly, after which 500 µl chloroform, 100 µl internal standard and 1 ml demi-water were added. After centrifugation the chloroform layer was collected and dried. The

remaining pellet was dissolved in 50 µl chloroform and put on a HPTLC plate. With HPTLC analysis, triglycerides, cholesterol and cholesterol esters were separated and the amount was quantified by scanning the plates with a Hewlett Packard Scanjet 4c and by integation of the density using Tina version 2.09 software (Raytest, Staubenhardt, Germany)

Real time quantitative PCR analysis

Total RNA was extracted from liver using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [24]. PCR primer sets (table 2) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. HPRT was used as the standard housekeeping gene. The significance of differences in relative gene expression numbers C_t ($C_{t(HPRT)}$–$C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a Mann-Whitney U test. Probability values less than 0.05 were considered significant.

Table 2. Primer sequences of genes used for mRNA quantification

Gene	*Forward primer*	*Reverse primer*
HPRT	5'-TTGCTCGAGATGTCATGAAGGA	5'-AGCAGGTCAGCAAAGAACTTATAG
mERα	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
ApoE	5'-AGCCAATAGTGGAAGACATGCA	5'-GCAGGACAGGAGAAGGATACTCAT
IL-6	5'-AAGAATTTCTAAAAGTCACTTTGAGATCTA	5'-CACAGTGAGGAATGTCCACAAAC
ApoAV	5'-GAGCAAAGGCGTGATGGG	5'-TGCTCGAAGCTGCCTTTCA
SHP	5'-CTATTCTGTATGCACTTCTGAGCCC	5'-GGCAGTGGCTGTGAGATGC
Cyp7A	5'-CTGTCATACCACAAAGTCTTATGTCA	5'-ATGCTTCTGTGTCCAAATGCC
PPARα	5'-CCTCAGGGTACCACTACGGAGT	5'-GCCGAATAGTTCGCCGAAA

Western blot analysis

Immunoblotting procedures were performed as described previously [25]. Livers of both Ad.Empty and Ad.shERα treated mice (n= 5) were lysed and homogenized in 200 µl of RIPA

buffer (1% NP40, 0.5% DOC, 0.1% SDS, 50mM Tris pH 8.0, 150mM NaCl, 2,5mM EDTA) containing protease inhibitor (40ul/ml, Roche). Extracts were cleared by centrifugation (4°C, 14 000 *g*, 5 min), and protein content was determined using the BCA kit (Pierce). Protein samples were denaturated (5 min, 90°C) and separated on SDS/PAGE by use of 8% gradient gels and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). Blots were stained with Ponceau S before blocking to verify equal loading and appropriate protein transfer. Membranes were blocked for 90 min in PBS, pH 7.4, containing 0.05% Tween 20 and 10% milk powder. Thereafter, membranes were incubated for 16 h at 4°C with ab MC20, 1:1000 (mERα rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). After extensive washing with blocking buffer without milk powder or BSA, membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000 (Promega). Membranes were again extensively washed and bound peroxidase conjugates were visualized by enhanced chemiluminescence (ECL, Amersham) on a LumiImager workstation. Additionally, filters were stripped by an 30 min incubation in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.8 at 50°C, to proceed with the whole procedure as described above. However, now membranes were incubated for 16 h at 4°C with p-38 ab, 1:1000 (N-20, cs-728, rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). Immunoblots were quantified using Lumianalyst software on a LumiImager (Boehringer-Mannheim).

Statistical analysis

Results are presented as mean ± SD values for the number of animals indicated. Differences between the experimental groups were determined by Mann-Whitney U test. The level of statistical significance of the difference was set at $P < 0.05$.

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4.

Administration of 17-β-estradiol to an insulin resistant mouse model acutely improves hepatic insulin sensitivity

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Abstract

Prolonged 17-β-estradiol (E_2) administration affects insulin sensitivity. However, it is unknown whether E_2 influences insulin sensitivity directly or indirectly e.g. via modulation of plasma free fatty acid levels and/or intra-hepatic lipid levels. Therefore, acute effects of E_2 administration were studied by performing a hyperinsulinemic-euglycemic clamp in an insulin resistant mouse model (APOE*3-Leiden mice, which had been fed a high fat diet for 13 weeks). Six hours after E_2 administration, estrogen receptor mediated transcription was induced predominantly in liver, but plasma triglyceride, insulin, free fatty acid and intra-hepatic lipid levels were unaffected. During the hyperinsulinemic clamp, the hepatic glucose production was significantly inhibited in the E_2 treated mice as compared to control mice (4.5±11 *versus* 34±29 $\mu mol \cdot min^{-1} \cdot kg^{-1}$; P=0.013), whereas the peripheral glucose disposal rate was significantly lower in the E_2 treated mice (29.8±8.9 *versus* 50.9±26.4 $\mu mol \cdot min^{-1} \cdot kg^{-1}$; P=0.017). The E_2-induced increased sensitivity of liver was accompanied by a significant decrease in the expression of hepatic genes involved in gluconeogenesis. Thus, administration of E_2 to an insulin resistant mouse model acutely improves hepatic insulin sensitivity at the expense of peripheral insulin sensitivity, through mechanisms independent of plasma lipid levels and hepatic lipid accumulation.

Introduction

17-β-estradiol (E_2) is a sex hormone that plays a major role in the establishment and maintenance of the reproductive tract and mammary glands [1-4]. In addition, E_2 is implicated in the regulation of a host of physiological processes including lipid and glucose metabolism [5-8]. E_2-deficiency, such as occurs after menopause in humans, is associated with many features of the metabolic syndrome including central obesity, insulin resistance and dyslipidemia (for review see [9]). Conversely, hormone replacement therapy has been associated with a reduction in the incidence of diabetes, which is a major complication associated with the metabolic syndrome [10,11]. However, it's obvious that menopause occurs as a function of aging, and aging itself is also associated with an increased incidence of the metabolic syndrome. Therefore, controversy remains regarding the role of E_2 in the metabolic syndrome.

More direct evidence for the effects of estrogens on glucose and lipid homeostasis has been obtained in mouse models. Ovariectomized mice become obese and insulin resistant [5]. Similarly, estrogen receptor α ($ER\alpha^{-/-}$) and aromatase knockout mice (ArKO, an estrogen-

deficient model) develop insulin resistance and impaired glucose tolerance [12-16]. However, also in these estrogen (receptor) deficient mouse models the glucose and lipid phenotypes require weeks to months to develop, implying involvement of many additional metabolic pathways. Thus, it is evident that estrogens have a multitude of effects on different processes in different organs, which apparently interact at multiple levels to achieve metabolic regulation. Consequently, it remains unclear to which extent the effects of E_2 on insulin sensitivity are the consequence of direct effects of E_2 and/or of indirect effects, e.g. related to long term effects of E_2 on triglyceride tissue distribution and ensuing tissue function.

To examine the short-term effects of E_2 on insulin sensitivity, we have studied the acute effects of E_2 in an insulin resistant mouse model. Male APOE*3-Leiden mice were used, which on a high-fat diet, develop many features of the metabolic syndrome, including obesity, hyperlipidemia, and insulin resistance [17]. Our results indicate that E_2 administration improves hepatic insulin sensitivity within several hours of administration at the expense of peripheral insulin sensitivity, independent of plasma and intra-hepatic lipid levels.

Results

Body weight and plasma parameters

To induce features of the metabolic syndrome, two groups of male APOE*3-Leiden mice were put on a high fat diet for a period of 13 weeks. This resulted in obese mice with a body weight of 35-37 grams. In addition, the mice exhibited hypercholesterolemia (4.5-4.7 mmol/l) and

Table 1. Plasma parameters in overnight-fasted APOE*3-Leiden mice fed a high fat diet that received E_2 or vehicle for 6 hrs

	Basal		**Hyperinsulinemic**	
	Vehicle	**6 hrs E_2**	**Vehicle**	**6 hrs E_2**
Bodyweight (gr)	35 ± 5	37 ± 3		
TG (mmol/l)	1.2 ± 0.6	1 ± 0.6		
Chol (mmol/l)	4.5 ± 1.6	4.7 ± 2.3		
Glucose (mmol/l)	7.9 ± 2.4	8.3 ± 1.6	8.9 ± 3.4	9.7 ± 2.8
Insulin (ng/ml)	1.5 ± 1.1	1.7 ± 1.1	4.8 ± 2*	5.6 ± 3.6*
FFAs (mmol/l)	1.4 ± 0.4	1.2 ± 0.5	0.9 ± 0.4*	0.7 ± 0.2*
GIR ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)			26.3 ± 14.2	25.7± 13.9

Plasma levels were measured during basal and clamp conditions. Body weight was measured just before the clamp. Values represent the mean ± SD of 8 mice per group. * p< 0.005 compared to basal conditions

moderate hyperinsulinemia (1.5-1.7 ng/ml) (table 1). Acute administration of E_2 did not affect body weight, plasma triglycerides (TG), cholesterol, insulin and free fatty acids (FFA) levels (table 2).

***ER activity* in vivo**

The biodistribution and peak activation time of E_2 after bolus injection depends on the type and site of administration and dissolvent used. To determine the tissues that are activated and time course of activation by E_2 in our hands, E_2-activity was monitored *in vivo* using a luciferase (luc) reporter system and a highly sensitive CCD camera. Non-invasive optical imaging was performed at different time points after E_2 injection in male transgenic reporter mice, in which the luc gene was driven by an estrogen response element (ERE) containing promoter (ERE-Luc mice), (Fig. 1). At time point t = 6 hours, *in vivo* luc expression peaked and was almost exclusively limited to liver. Therefore, the t = 6 hours after treatment point was taken as the moment to assess the acute effects of E_2 administration.

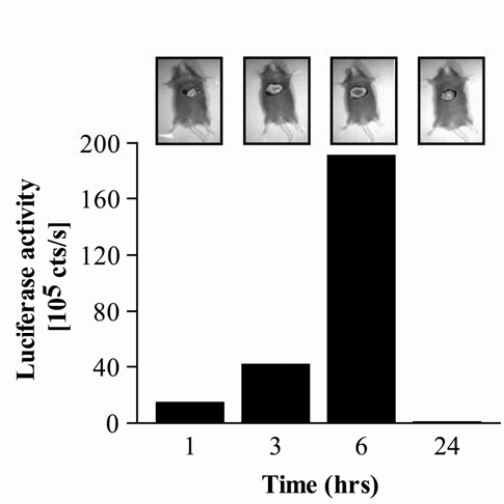

Figure 1. E_2 induced ER mediated transcription in ERE-Luc transgenic male mice.
Optical imaging of bioluminescence emitted from E_2 (50 μg/kg, sc) treated ERE-Luc transgenic male mice in time

Insulin sensitivity after E_2-treatment

To determine the acute effect of E_2 administration on insulin sensitivity, a hyperinsulinemic-euglycemic clamp study was performed six hours after E_2 administration. During hyperinsulinemic conditions, no significant differences were observed in plasma glucose levels (table 2). FFA levels were suppressed (P = 0.002) to a similar extent in both vehicle and E_2 treated mice (table 2). The clamp results showed a significantly lower insulin-mediated whole-body glucose uptake in the E_2 treated mice as compared to control mice (29.8±8.9 *versus* 50.9±26.4 μmol · min^{-1}. kg^{-1}; P=0.017; Fig 2A). Moreover, no significant insulin-mediated suppression of hepatic glucose production (HGP) was observed in control mice, indicative for a state of hepatic insulin resistance. In contrast, in the E_2 treated mice, HGP was significantly suppressed under

hyperinsulinemic conditions, from 39.3±11.5 to 4.5±11.2 μmol · min^{-1} · kg^{-1} (P=0.0002; Fig 2B). Thus, acute E_2 treatment attenuates peripheral insulin sensitivity with regard to glucose disposal, but improves hepatic insulin sensitivity with regard to suppression of HGP.

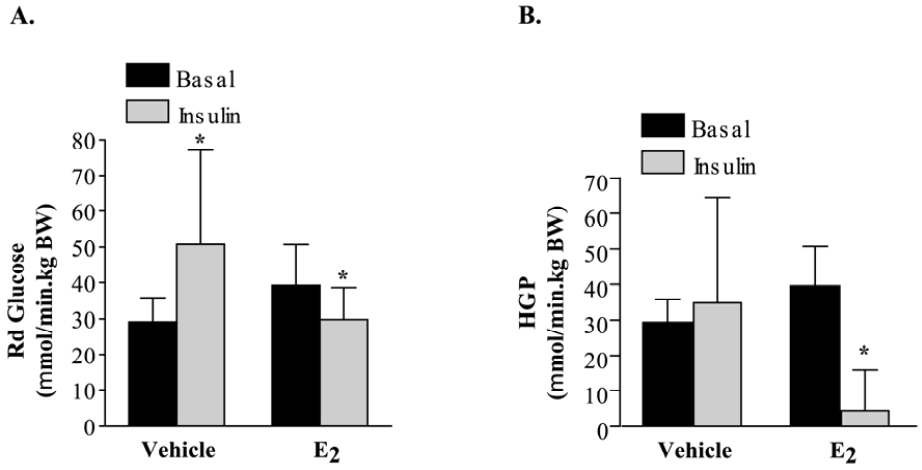

Figure 2. Peripheral and Hepatic insulin sensitivity E_2 treated APOE*3-Leiden mice
Hyperinsulinemic-euglycemic clamp of APOE*3-Leiden male mice six hours after vehicle or E_2 (100 μg/kg, sc) treatment. (A) Basal and insulin-mediated stimulation of whole-body glucose uptake (B) Basal and insulin-stimulated rates of HGP. Data are means±SD, n=8. *P<0.05, using nonparametric Mann-Whitney tests.

Hepatic lipid content after E_2 treatment

Male APOE*3-Leiden mice on a high-fat diet develop steatosis, which may be causally related to hepatic insulin resistance. Since hepatic insulin sensitivity improved acutely after E_2 treatment, hepatic TG and cholesterol content were analyzed. As depicted in Fig 3, the E_2 treated mice did not exhibit a change in hepatic lipid content compared to the vehicle treated group (cholesterol content: 17.8±5.1 *versus* 21±6.8 μg/mg; TG content: 106±16.8 *versus*

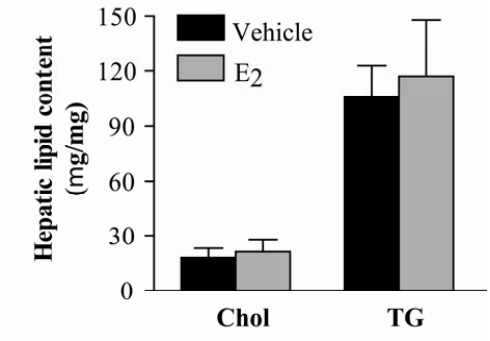

Figure 3. Effect acute E_2 administration on hepatic lipid content.
Hepatic TG and cholesterol content was analyzed under hyperinsulinemic conditions in E_2 versus control treated APOE*3-Leiden male mice. Values represent the mean±SD of 8 mice. *, statistically significant difference of P<0.05 compared with vehicle treated mice.

117±30.9 μg/mg, respectively) indicating that E_2 improves hepatic insulin sensitivity independently of a change in hepatic lipid content.

Hepatic mRNA expression levels

To examine the improved sensitivity of the liver to insulin-mediated inhibition of HGP in more detail, mRNA levels of relevant genes were analyzed by taqman analysis. Short-term induction of hepatic ER activity led to a 2.3- fold reduction of hepatic peroxisomal proliferators-activated receptor-γ coactivator-1α (PGC-1α) RNA levels (P = 0.0002) (Fig 4A). The expression of PhosphoEnolPyruvateCarboxyKinase (PEPCK) and Glucose-6-phosphatase (G6P) were unchanged (Fig 4A). In addition the expression of Glycogen Phosphorylase (GP) was 1.7-fold decreased (P=0.0016) (Fig 4A).

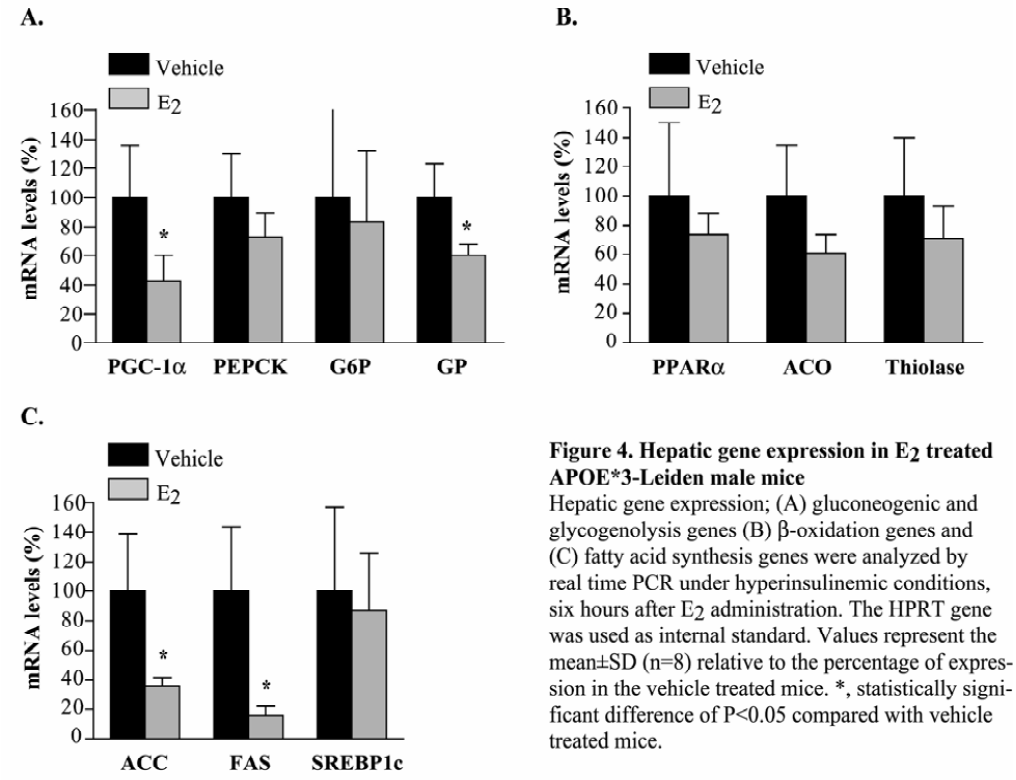

Figure 4. Hepatic gene expression in E_2 treated APOE*3-Leiden male mice

Hepatic gene expression; (A) gluconeogenic and glycogenolysis genes (B) β-oxidation genes and (C) fatty acid synthesis genes were analyzed by real time PCR under hyperinsulinemic conditions, six hours after E_2 administration. The HPRT gene was used as internal standard. Values represent the mean±SD (n=8) relative to the percentage of expression in the vehicle treated mice. *, statistically significant difference of P<0.05 compared with vehicle treated mice.

Since the hepatic glucose pathway is linked to the hepatic lipogenic pathway and since it has been shown that long-term modulation of E_2 signaling affects expression of enzymes involved in fatty acid β-oxidation and fatty acid synthesis, genes involved in these pathways were also analyzed. The expression of PPARα, Acyl-CoA oxidase (ACO, catalyzing the initial step of peroxisomal β-oxidation) and thiolase (catalyzing the final step of β-oxidation) were not significantly affected by acute E_2 administration (Fig 4B). On the other hand, acetyl CoA carboxylase α (ACCα) and fatty acid synthase (FAS), both key enzymes involved in *de novo* synthesis of fatty acids were significantly repressed (6.3- and 2.8-fold, respectively), while the sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor able to activate lipogenic genes like FAS and ACCα was unchanged (Fig 4C).

Discussion

The present study demonstrates for the first time that E_2 acutely improves hepatic insulin resistance with regard to HGP, whereas it attenuates peripheral insulin sensitivity with regard to glucose disposal in an insulin resistant mouse model. The improvement in hepatic insulin resistance was associated with decreased hepatic expression of the transcription factor PGC-1α and the glycogenolysis enzyme GP. Simultaneously, plasma FFA levels and hepatic lipid content were not affected. Thus E_2 has acute effects on hepatic insulin sensitivity, independent of hepatic lipid accumulation.

Our results show that there are specific differences with respect to insulin sensitivity between acute and long-term administration of E_2. In rat and mice models, long-term E_2 modulation results in an improvement of both hepatic and peripheral insulin sensitivity with respect to glucose output and disposal [16,18,19]. In contrast, short-term E_2 administration improves hepatic insulin sensitivity but attenuates peripheral insulin sensitivity. Since long-term E_2 administration does affect TG distribution and body weight, it is possible that the long-term effects of E_2 on peripheral insulin sensitivity are a long term consequence of these physiological changes. Specifically, the preventive effect of estrogen on steatosis would positively affect hepatic insulin sensitivity, since steatosis and hepatic insulin resistance are highly correlated. However, since E_2 will affect many organs and also affects neuro-endocrine signaling, it is likely that the net effect of long-term E_2 administration is the result of multiple complex interactions.

The APOE*3-Leiden mice, fed a high fat diet for 13 weeks, are highly resistant to insulin-mediated suppression of HGP, as demonstrated by no reduction in HGP by insulin

(Fig 2B). In comparison, chow-fed age-matched APOE*3-Leiden mice show 40-50% suppression of HGP by insulin. The single dose of E_2 acutely increased the insulin-mediated suppression of HGP in the fat-fed APOE*3-Leiden mice at least to the level of chow-fed APOE*3-Leiden mice. Thus a single dose of E_2 has a highly potentiating effect on insulin sensitivity of HGP.

Peripheral glucose disposal is approximately 150% increased by insulin in chow-fed age-matched APOE*3-Leiden mice. In high fat-fed APOE*3-Leiden mice it is only increased by 50%. Thus peripheral glucose disposal is also insulin resistant. This situation is further deteriorated by administration of E_2. At the moment, we have no explanation for this phenomenon. Since acute E_2 treatment did not reveal ER activity in muscle and adipose tissue in living ERE-Luc reporter mice, is seems likely that non-transcriptional E_2-mediated processes play a role. However, it is also possible that the bioluminescence method is not sensitive enough to detect limited, but potentially physiologically relevant ER activation in skeletal muscle and adipose tissue.

Under physiological insulin sensitive conditions, insulin reduces HGP through inhibition of hepatic gluconeogenesis and glycogenolysis. In contrast, under insulin resistant conditions, insulin is unable to suppress HGP and this has been associated with a failure in the down regulation of hepatic genes involved in glucose output [20]. In the present study, acute administration of E_2 in insulin resistant mice did not change expression levels of G6P and PEPCK (Fig 4A), which both play important roles in gluconeogenesis. However, the transcriptional coactivator protein PGC-1α, identified as an important inducer of gluconeogenesis [21], was significantly reduced upon acute administration of E_2 (Fig 4A). This apparent discrepancy indicates that under these conditions, down regulation of PGC-1α is not a dominant effect in the down regulation of G6P and PEPCK gene expression. More in line with the observed effect of E_2, expression of GP, an enzyme involved in glycogenolysis was significantly decreased. Whether this effect is directly related to down regulation of PGC-1α remains to be determined. Our data demonstrate that acute E_2 treatment regulates hepatic glucogenic genes, which could at least partly explain the observed E_2 mediated improvement of insulin-mediated inhibition of HGP.

Acute E2 administration did not affect intrahepatic TG levels in high fat-fed APOE*3-Leiden mice. In contrast, long term modulation of E_2 signaling is known to affect intrahepatic lipid levels. For example, constitutive E_2 deficiency, such as occurs in male aromatase knockout mice results in severe hepatic steatosis [13]. This phenotype has been associated

with decreased expression of genes involved in fatty acid β-oxidation [22,23] such as ACO and Thiolase, but also with increased expression of genes involved in de novo synthesis of fatty acids, including FAS and ACCα [13].

We found that acute E_2 treatment in insulin resistant APOE*3-Leiden did not change mRNA levels of genes involved in hepatic β-oxidation (Fig 4B). However, the fatty acid synthesis genes, FAS and ACCα were clearly suppressed (Fig 4C). Nevertheless, E_2 mediated suppression of FAS and ACCα did not result in decreased intrahepatic TG levels. It is possible that the six hour time window in the current study may not be sufficient to detect differences in intrahepatic TG flux. Alternatively, the effects of reduced FAS and ACCα gene expression are overruled by other compensatory transcriptional and/or post-transcriptional mechanisms.

In conclusion, administration of E_2 results in an acute improvement of hepatic insulin sensitivity with respect to HGP in obese, hyperlipidemic and insulin resistant mice fed a high-fat diet. This effect is independent of body weight, plasma lipid levels and/or intrahepatic TG content. Concomitantly, the administration of E_2 acutely impairs peripheral insulin sensitivity, through mechanisms not understood. These data demonstrate that acute and long-term administration of E_2 differentially affects tissue-specific insulin sensitivity.

Methods

Animals

All animal work was approved by the Animal Ethic Committee from the Leiden University Medical Center and TNO-Prevention and Health, Leiden, the Netherlands and the experimental protocols complied with the national guidelines for use of experimental animals. 32 Wks old APOE*3-Leiden male mice (n=16) generated in the animal facility of TNO-Prevention and Health, were housed under standard conditions in conventional cages with free access to water and food. At the age of 19 wks, they were fed a high fat diet containing 45.4% fat (Hope Farms, Woerden, The Netherlands) for 13 wks.

Bioluminescent reporter imaging.

The experiment was carried out in male transgenic reporter mice, in which the luciferase gene was driven by an estrogen response element containing promoter (ERE-Luc mice) [24]. At time point 1, 3, 6 and 24 hours after s.c injection of 100 μg/kg 17 β-estradiol (dissolved in sesam oil, Sigma) in the neck, bioluminescent signals (BLS) were measured by Xenogen

IVIS imaging system (IVIS 100). The living mice were intraperitoneally injected with the luciferase substrate, luciferin, at a dose of 150 mg/kg body weight approximately 5 minutes before imaging. The mice were anaesthetized with isoflurane/oxygen and placed on the imaging stage. Total photon emission of each animal was acquired for 1 minute. Captured images were quantified by using the Living Image software (Xenogen Corp, Almeda, CA) and the IGOR software (WaveMetrics Corp, Lake Oswego, OR). BLS from the region of interest (ROI) was expressed using the pseudo colour scale (Red most intense and Blue least intense luminescence) and the data were presented as the cumulative photon counts collected within each ROI.

Plasmaparameters

Blood samples were taken via tail bleeding in paraoxon-coated capillaries, to prevent lipolysis [25]. Plasma was collected by centrifugation at 4°C. Plasma levels of total Chol, TG and FFAs were determined enzymatically using commercially available kits and standards (Sigma Diagnostics, St. Louis, MO; Roche Molecular Biochemicals GmbH, Mannheim, Germany; and Wako Chemicals GmbH, Neuss, Germany). Plasma insulin was measured by ELISA (Mercodia Ultrasensitive mouse insulin ELISA, Mercodia, Sweden). Levels of plasma glucose were determined were determined using a commercially available kit (Sigma; Boehringer Mannheim, Mannheim, Germany). During the clamp experiment, whole-blood glucose was measured by a Freestyle hand glucose analyzer (Disetronic, Vianen, The Netherlands). All plasma parameters were determined according to the manufacturers' instructions.

Hyperinsulimemic euglycemic clamp

Male APOE*3-Leiden mice fed a high fat diet, fasted overnight (food withdrawn at 05.00 hour p.m.) were given 17β-estradiol (s.c, 100μg/kg) (Sigma, E8875) (n=8) or vehicle (sesame oil, Sigma) (n=9) at 06.00 hour a.m. Six hours after treatment insulin-mediated suppression of endogenous (hepatic) glucose production was studied by performing a hyperinsulinemic-euglycemic clamp analysis using ^{3}H-D-Glucose as tracer. The clamp analysis and calculations were performed as described previously [26]. After the last blood sample, mice were sacrificed, livers were taken out and immediately frozen using liquid nitrogen and stored at -80°C until further analysis.

Calculations

Total plasma ^{3}H-glucose radioactivity was determined in10-µl plasma and in supernatants after trichloric acid (20%) precipitation and water evaporation to eliminate [^{3}H]-H_2O. The rates of glucose oxidation were determined as previously described [27]. Under steady state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance (Ra; *i.e.* endogenous glucose production plus exogenous D-glucose infusion). Ra glucose was calculated as the ratio of the rate of infusion of [3-^{3}H] glucose (dpm) and the steady-state plasma [^{3}H] glucose specific activity (dpm/µmol glucose). The hepatic glucose production was calculated as the difference between the rate of glucose disappearance and the infusion rate of exogenous D-glucose.

Hepatic lipid analysis

Liver samples were homogenized in PBS. Protein content was measured by BCA protein assay kit (Pierce) at 562 nm. Hepatic lipids were extracted according to Bligh and Dyer [28]. After dissolving the lipids in 2% Triton X-100, the contents of Chol and TG in liver tissues were determined as described above.

Table 2. Primer sequences of genes used for mRNA quantification

Gene	*Forward primer*	*Reverse primer*
HPRT	5'-TTGCTCGAGATGTCATGAAGGA	5'-AGCAGGTCAGCAAAGAACTTATAG
FAS	5'-GGCATCATTGGGCACTCC	5'-GCTGCAAGCACAGCCTCT
ACC1	5'-GCCATTGGTATTGGGGCT	5'-CCCGACCAAGGACTTTGTT
SREBP1c	5'-GGAGCCATGGATTGCACA	5'-CCTGTCTCACCCCCAGCA
mERα	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
PEPCK	5'-CCATGAGATCTGAGGCCACA	5'-GTATTTGCCGAAGTTGTAGCCG
G6P	5'-CAGGTCGTGGCTGGAGTCTT	5'-GACAATACTTCCGGAGGCTGG
GP	5'-GCGGTGACCGGTGTAGCAA	5'-CTTGTCTGGTTCTAGCTCGCTG
PGC1	5'-TTTTTGGTGAAATTGAGGAATGC	5'-CGGTAGGTGATGAAACCATAGCT
PPARα	5'-CCTCAGGGTACCACTACGGAGT	5'-GCCGAATAGTTCGCCGAAA
ACO	5'-GCCACGGAACTCATCTTCGA-3'	5'-CCAGGCCACCACTTATGGA-3'
Thiolase	5'-GGCAGGTTGTCACGCTACTCA-3'	5'-ATGGATACCACGCCGTAAGC-3'

Real time quantitative PCR analysis

Total RNA was extracted from liver using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse

transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [29]. PCR primer sets (table 2) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. Since HPRT did not respond to the estrogen treatment, it was used as the standard housekeeping gene. The differences in relative gene expression numbers was calculated by C_t ($C_{t(HPRT)}$–$C_{t(target\ gene)}$). The date was verified by use of another independent housekeeping gene, cyclophilline.

Statistical analysis

Results are presented as mean ± SD values for the number of animals indicated. Differences between the experimental groups were determined by Mann-Whitney U test. Probability values less than 0.05 were considered significant.

Acknowledgements

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Efficient targeting of adenoviral vectors to integrin positive vascular cells utilizing a CAR-cyclic RGD linker protein.

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Abstract

Vascular smooth muscle (VSMC) and endothelial cells (EC) are particularly resistant to infection by type 5 adenovirus (Ad) vectors. To overcome this limitation and target Ad vectors to ubiquitously expressed $\alpha_V\beta_{3/5}$ integrins, we have generated a linker protein consisting of the extra cellular domain of the coxsacky adenovirus receptor (CAR) connected via avidin to a biotinylated cyclic (c) RGD peptide. After optimization CAR to cRGD and to Ad coupling, infection of mouse heart endothelial cells (H5V) could be augmented significantly, as demonstrated by 600-fold increased transgene expression levels. In EOMAs, a hemangioendothelioma-derived cell line, the fraction of infected cells was enhanced 4-6 fold. Furthermore, the fraction of infected primary mouse VSMC was increased from virtually 0 to 25%. Finally, in human umbilical vein endothelial cells (HUVECs), the number of GFP positive cells was enhanced from 2% to 75%. In conclusion, CAR-cRGD is a versatile and highly efficient construct to target Ad vectors to both transformed and primary VSMC and EC.

Introduction

Recombinant type 5 adenovirus (Ad) vectors are extensively used to modulate gene expression in a wide variety of cells and organs, both *in vitro* and *in vivo*. Part of this popularity can be ascribed to their relatively straightforward generation and amplification to high titers [1]. Ad entry and infection of cells requires at least two distinct interactions. First, attachment of the virus particle occurs via interaction of its fiber knob with the coxsacky adenovirus receptor (CAR) present on the cell surface [2-5]. Second, the Arg-Gly-Asp (RGD) motifs present in the viral penton base will bind to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the target cell surface and trigger internalization via receptor-mediated endocytosis [6-8]. In addition, recent data have shown the involvement of heparan sulfate glycosaminoglycans (HSGs) in adenoviral entry *in vivo* [9]

Recombinant Ad vectors encoding numerous wild type and mutant genes, as well as short hairpin RNA molecules have been generated. However, the application of Ad vectors in CAR negative cell lines, such as vascular smooth muscle cells (VSMC) and endothelial cells (EC) [10-12], is hampered by low infection efficiencies at low multiplicity of infection (MOI) and Ad associated cytotoxicity at high MOI.

To expand the applicability of Ad-mediated gene transfer, various strategies to modify Ad tropism have been undertaken. In the genetic modification approach, peptide ligands have

been incorporated into the HI-loop of the Ad fiber knob [13-16], added to the C-terminus of the fiber knob [17] or inserted into the hexon protein [18]. However, it is not possible to predict which peptide or protein ligands will be tolerated and do not disturb fiber trimerization and/or capsid function. In addition, for each specific targeting application, rederivatization of the original recombinant Ad vectors is obligatory. Alternatively, bifunctional targeting proteins have been generated consisting of an Ad-binding domain coupled to a peptide or protein that confers a novel specificity [19]. This strategy enables the utilization of existing recombinant Ad vectors, but the generation of the bifunctional targeting protein may require chemical linkage and subsequent purification steps. In addition, Parrot and co-workers have introduced a novel approach to target viral vectors. They launched the concept of metabolically biotinylated vectors [20,21] and demonstrated the utility of the avidin-biotin based system for vector targeting.

Recently we have combined the advantages of the latter two targeting strategies, by developing a bi-functional linker protein that exploited the avidin-biotin concept (Gras, personal communication). This linker protein consists of the extra cellular domain of the CAR fused to chicken avidin, which functions as a universal docking site for biotinylated ligands. It was demonstrated that a biotinylated dA_6G_{10} oligonucleotide coupled to the CAR-Avidin linker confers macrophage specificity (Gras, personal communication). In this study, the CAR-Avidin linker protein is coupled to a biotinylated cyclic RGD peptide (bio-cRGD) to increase infection efficiency of EC and VSMC. This cRGD peptide has a high affinity for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins [22], which are expressed ubiquitously on transformed cell lines and most primary cells. It is demonstrated that linking of Ad to the CAR-cRGD targeting construct resulted in a highly significant improvement of infection efficiencies of transformed and primary VSMC and EC at all MOI used.

Results

Generation and optimization of the adenovirus targeting construct

To target Ad to $\alpha_V\beta_{3/5}$ integrins, the bi-functional linker protein CAR-Avidin was equipped with the targeting peptide bio-cRGD to yield CAR-cRGD. The optimal ratio of CAR-Avidin to bio-cRGD, resulting in a complete occupation of all available biotin binding sites was determined by a ^{3}H-biotin binding assay. Figure 1A shows the amount of ^{3}H-biotin that is still able to bind to CAR-Avidin at a molar ratio of CAR-Avidin to bio-RGD ranging from 1:0.001 to 1:3. At a molar ratio of more than 1:0.3, no residual ^{3}H-biotin binding capacity could be detected, indicating that at this ratio all biotin-binding sites of CAR-Avidin

were occupied. In all following experiments, a slight excess of CAR-Avidin to bio-cRGD was used (ratio 1:1) to generate the CAR-cRGD targeting construct.

To determine the optimal ratio of Ad to CAR-cRGD targeting construct, a fixed amount of Ad.LacZ was incubated with various concentrations of CAR-cRGD and added to $\alpha_V\beta_{3/5}$-positive mouse heart endothelial cells (H5V) [23]. Two days after infection, the cells were fixed and stained for β-galactosidase activity. The number of lacZ positive cells was enhanced in a CAR-cRGD concentration dependent manner (Fig. 1B) ranging from 1-270 positive cells per microscope field. The increase in infection efficiency leveled off at a concentration of 1.2 μM CAR-cRGD. Therefore in the subsequent experiments, a concentration of 1.2 μM CAR-cRGD was used.

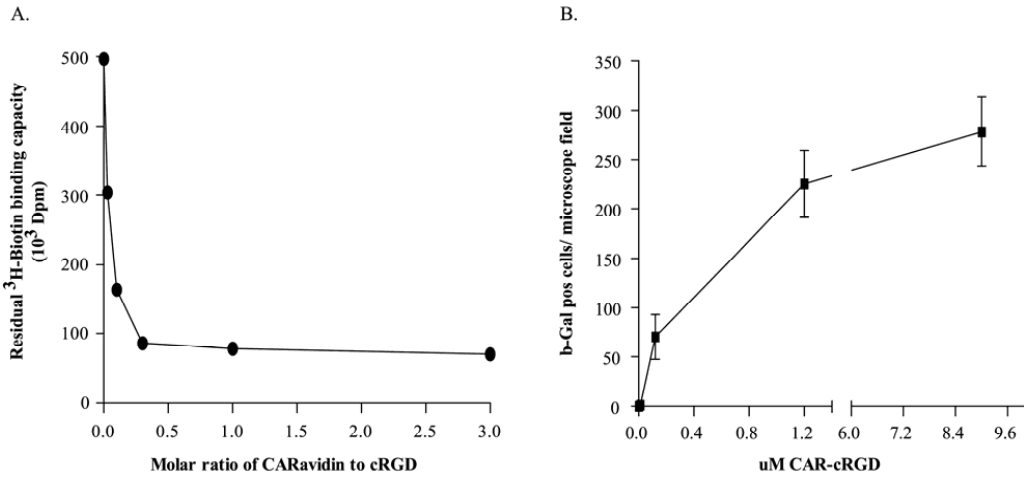

Figure 1. Optimization of Ad : CAR-Avidin / cRGD ratio
(A) CAR-Avidin (30 nM) preincubated with different molar ratios of biotin-cRGD was incubated with an excess of ^{3}H-biotin. CAR-biotin (-^{3}H or -cRGD) radioactivity was counted. (B) Ad.LacZ was preincubated with either BSA (Ad) or with different amounts of the targeting construct, CAR-cRGD (cRGD-Ad) and subsequently added at a titer of MOI 1000 to mouse endothelial cells for 1 hr. Forty hrs post infection, cells were fixed and stained for β-galactosidase activity for 4 hrs. Multiple microscope fields were counted for positive cells. Values represent mean ± SD of three samples.

cRGD mediated adenoviral gene transfer in CAR deficient and $\alpha_v\beta_{3/5}$ positive cells

The ability of the CAR-cRGD targeting construct to achieve CAR-independent gene transfer was determined in the CAR-negative, but $\alpha_V\beta_{3/5}$ positive cell line CHO [24]. Near confluent CHO cells were infected with untargeted Ad versus targeted Ad-vectors expressing luciferase (cRGD-Ad.Luc) (MOI 100-2500). 40 hrs after infection, cell lysates were evaluated

for luciferase expression (Fig 2A). As expected, Ad.Luc was incapable of infecting CHO cells even at an MOI of up to 500. In contrast, already at MOI 100, the cRGD-Ad.Luc vector resulted in a 3 to 4 log-fold enhancement of luciferase expression, demonstrating that the cRGD- equipped Ad.Luc achieves gene transfer through a CAR-independent cell entry pathway.

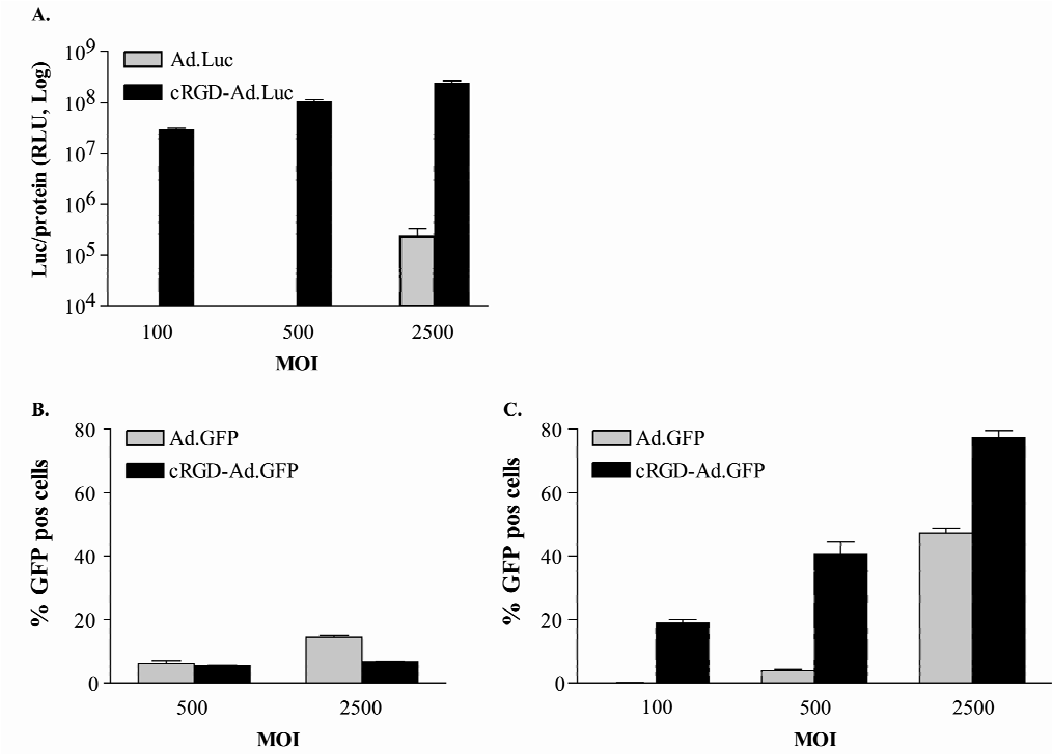

Figure 2. Analysis of αVβ3-/αVβ5-integrin and CAR dependent gene transfer of cRGD equipped Ad
(A) CAR-negative CHO cells were exposed for 1 hr to Ad.luc, preincubated with BSA (Ad) or 1200 nM CAR-cRGD (1:1M) (cRGD-Ad) at different MOI. Forty hrs after infection luciferase expression was measured and corrected for protein levels. Values represent mean ± SD of three samples. (B) Ramos and (C) K-562 cells were infected with an increasing titer of unmodified Ad (Ad.GFP) or cRGD equipped Ad-vector (cRGD-Ad.GFP). CAR-Avidin was prebound to cyclic RGD at a 1:1 molar ratio and 1200 nM of the complex was incubated with Ad. 24 hrs after infection FACs analysis was performed. Values represent the mean ± SD of three samples

The specificity of targeting Ad to $\alpha_V\beta_{3/5}$ integrins was investigated by comparing gene transfer of Ad versus cRGD-equipped Ad in human leukemia cell lines, Ramos and K-562. In both cell lines moderate levels of CAR are present, however only K-562 cells express $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins [25]. Ad.GFP plus or minus cRGD was applied to both cell lines at an MOI ranging from 100 to 2500. After 24 hrs, the cells were monitored for GFP expression by

FACS analysis (Fig 2B and 2C). In both the $\alpha_V\beta_{3/5}$ integrin negative as well as positive cell line, gene transfer mediated by unmodified Ad.GFP was low but dose-dependently increased. In Ramos cells, which do not express $\alpha_V\beta_{3/5}$ integrins, cRGD-mediated gene transfer did not increase infection efficiency at MOI 500, as compared to unmodified Ad. Moreover, at MOI 2500 cRGD-mediated gene transfer remained low, resulting in a significantly lower percentage of GFP positive cells as compared to unmodified Ad.GFP. In contrast, in the $\alpha_V\beta_{3/5}$ integrin positive K-562 cell line, cRGD-mediated gene transfer resulted in an approximately 10-fold increase in the number of GFP positive cells as compared to those infected with untargeted Ad.GFP at all MOIs used. Thus, cRGD-Ad markedly enhanced gene transfer only in the $\alpha_V\beta_{3/5}$ integrin positive K-562 cell line, suggesting that the $\alpha_V\beta_{3/5}$ integrins are involved in the uptake of cRGD equipped Ad vectors.

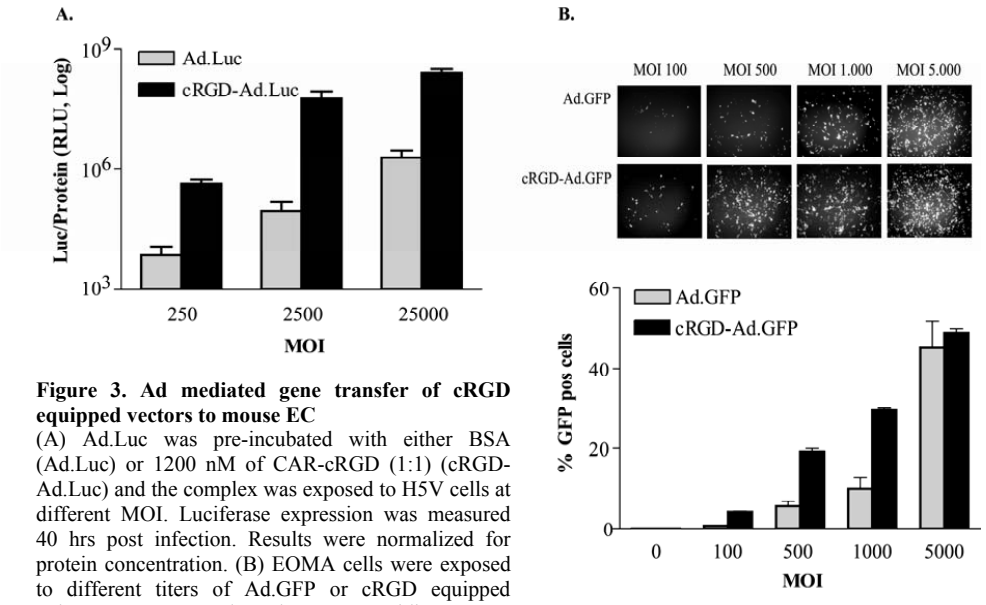

Figure 3. Ad mediated gene transfer of cRGD equipped vectors to mouse EC
(A) Ad.Luc was pre-incubated with either BSA (Ad.Luc) or 1200 nM of CAR-cRGD (1:1) (cRGD-Ad.Luc) and the complex was exposed to H5V cells at different MOI. Luciferase expression was measured 40 hrs post infection. Results were normalized for protein concentration. (B) EOMA cells were exposed to different titers of Ad.GFP or cRGD equipped Ad.GFP. cRGD was bound to CAR-Avidin at a 1:1 molar ratio and 1200 nM of the CAR-cRGD conjugate was incubated with Ad.GFP. Values represent mean ± SD of three samples

Quantification of targeting efficiency in transformed vascular cell lines

The optimized targeting conditions were used to determine the efficiency of Ad-mediated gene delivery to murine vascular cell lines. Mouse heart endothelial (H5V) cells [26] were incubated with increasing concentrations of Ad.Luc or cRGD-Ad.Luc. Luciferase expression levels showed a titer-dependent increase. As compared to gene delivery with untargeted Ad.Luc, cRGD-Ad.Luc showed a 59-fold increased luciferase expression at MOI

250 and a 650-fold increased luciferase expression at MOI 2500 (Fig. 3A). To verify these results, the experiment was reproduced in a second mouse cell line, the hemangioma-derived

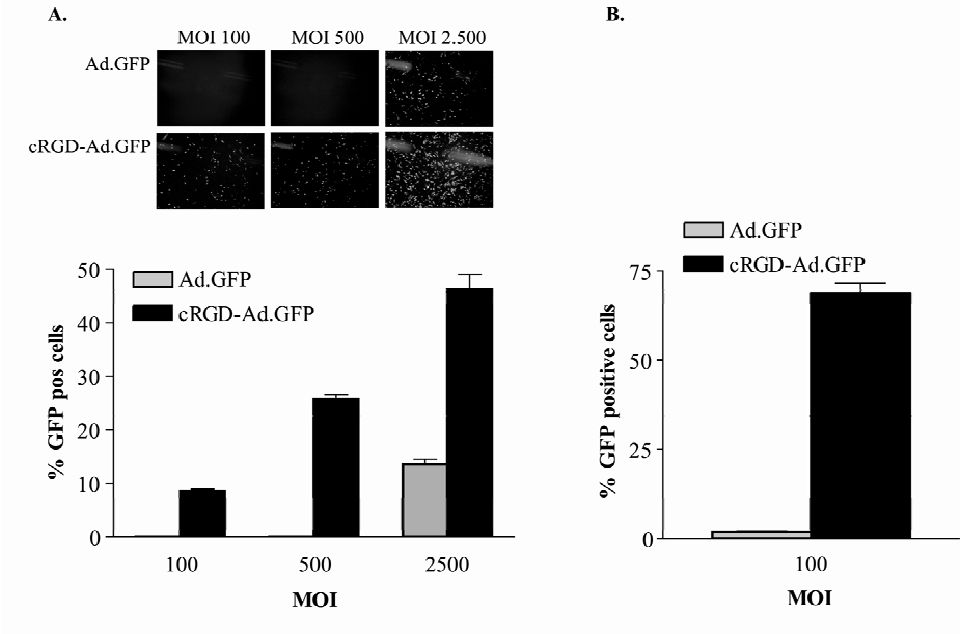

Figure 4. Ad mediated gene transfer of cRGD equipped vectors to primary cells
(A) Mouse vascular smooth muscle cells and (B) HUVECs were exposed for 1 hr to different MOI of Ad.GFP or to cRGD-Ad.GFP. CAR-Avidin was bound to cyclic RGD at a 1:1 molar ratio and 1200 nM of this complex was incubated with Ad.GFP. Forty hrs after infection FACs analysis was performed. Values represent mean ± SD of three samples

Quantification of targeting efficiency in primary Mouse VSMs and Human EC

Next, cRGD-mediated Ad targeting to primary vascular cells was examined. Primary mouse VSMC isolated from aorta and HUVECs were infected with either Ad.GFP or Ad.GFP equipped with the CAR-cRGD construct. Two days after infection, FACS analysis was performed to determine GFP expression levels. Primary VSMC were highly resistant to infection, as only 0.02% of the cells were infected with untargeted Ad.GFP (MOI 500) and 13% at high MOI (2500). In contrast, cRGD-Ad.GFP mediated gene transfer resulted in a titer-dependent increase in GFP positive cells up to 46.5% at MOI 2500 (Fig 4A). At MOI 500, this amounted to a 25-fold increased infection efficiency of primary VSMC using cRGD equipped Ad-vectors. Improvement of gene transfer was also tested in HUVECs. Gene transfer using untargeted Ad.GFP resulted in a very low percentage of GFP positive cells

(2%), while cRGD targeting of Ad led to a 36-fold increase (MOI 100) of infected HUVECs (Fig 4B).

Discussion

In the present study, we show that coupling of recombinant Ad vectors to a CAR-cRGD linker protein results in a significantly improved infection efficiency of both transformed and primary VSMC and EC. Conjugation of CAR-cRGD to Ad reporter vectors markedly enhanced gene transfer to the established endothelial cell lines H5V and EOMA (up to 59-fold), as well as to primary HUVEC (36-fold) and VSMC (25-fold). This was associated with a considerable increase in the percentage of infected cells. Thus, the CAR-cRGD targeting construct expands the utility of Ad vectors to CAR-negative cell types that do express $\alpha_V\beta_3$- and $\alpha_V\beta_5$ integrins.

The biotin-avidin based coupling of a ligand to the CAR adaptor molecule is straightforward and highly efficient due to the femtomolar affinity of biotin for avidin [27,28]. As compared to chemical modifications, this obviates the use of complex reaction mixtures and purification steps and enables simple quantification of CAR adaptor concentrations and optimal CAR-Avidin / biotin-cRGD ratios by a ^{3}H-biotin binding assay. Moreover, the CAR-Avidin adaptor may be coupled to a wide variety of biotinylatable ligands and has recently been successfully applied to target Ad vectors to macrophages using a biotinylated oligonucleotide (Gras, personal communication).

Application of cRGD-Ad vectors expressing GFP increased the number of infected vascular cells rather than that it boosted gene expression in a limited cell population, as compared with untargeted Ad (Figs 3B, 4A and 4B). Thus, CAR-cRGD mediated targeting of recombinant Ad vectors allows the use of considerably reduced MOIs to obtain near-quantitative gene transfer, thereby decreasing the vector related toxicity. This is particularly important for those cells that are sensitive to Ad-mediated toxicity. For example, only 2% of HUVECs were infected with unmodified Ad.GFP at MOI 100, whereas the same titer of cRGD equipped Ad.GFP resulted in 75% infected cells (Fig. 4B). To obtain a similar level of infection with unmodified Ad.GFP, MOI's >1000 would be required, which coincides with cytotoxicity (data not shown). In addition to a lower virus dose, the near quantitative infection of HUVECs enables the application of Ad vectors encoding inserts that require quantitative infection, such as short hairpin (sh)RNA constructs to knockdown gene function.

$\alpha_V\beta_3$- and $\alpha_V\beta_5$ integrins are known to be up-regulated on proliferating EC and subsequently have been exploited as targets to develop anticancer drugs. For this purpose, linear- and cyclic RGD peptides have been developed and used as a targeting moiety to selectively deliver drugs to angiogenic blood vessels [29,30]. Pfaff et al. have shown that a cyclic RGD peptide displayed a higher affinity for $\alpha_V\beta_3$- and $\alpha_V\beta_5$ integrins than the linear RGD peptide [22]. We have confirmed this observation, showing a 4-fold increased β-galactosidase activity utilizing cRGD-Ad vectors compared to linear RGD equipped Ad vectors (data not shown). The mechanism of cRGD-Ad mediated gene transfer was further characterized by infection of cell lines that differ in their expression levels of $\alpha_V\beta_{3/5}$ integrins and CAR. CHO and K-562 cells, which express $\alpha_V\beta_{3/5}$ integrins but not CAR, were efficiently transduced by cRGD-Ad vectors. On the other hand, Ramos cells, which do not express $\alpha_V\beta_{3/5}$ integrins, were almost completely resistant to infection by cRGD targeted Ad vectors. These results demonstrated that the entry route of our CAR-cRGD targeted Ad vectors is CAR independent and most likely mediated via $\alpha_V\beta_{3/5}$ integrins. Conversely, in all these three cell lines very low infection efficiencies were obtained for untargeted Ad vectors. At the high MOI of 2.500, only the integrin expressing cell lines, CHO and K-562 were infectable. Apparently, at this very high MOI, the local concentration of Ad particles was high enough to bind via their RGD motifs present in the viral penton base to the $\alpha_V\beta_{3/5}$ integrins and trigger internalization.

In several cell types and tissues which represent important targets for gene therapy, like the vascular system, the expression level of the endogenous adenovirus receptor CAR is low [31-33]. On the other hand, $\alpha_v\beta_{3/5}$ integrins are abundantly expressed on activated and proliferating EC and VSMC, which are present during angiogenesis, neovascularization, and inflammation [34-36]. *In vitro* the majority of proliferating cells express $\alpha_v\beta_{3/5}$ integrins. Therefore, the bifunctional linker protein carrying specificity for Ad vectors on the one hand and for $\alpha_V\beta_{3/5}$ integrins on the other hand greatly expands the applicability of conventional Ad vectors. In addition to providing Ad vectors with a novel tropism, the CAR.cRGD construct likely prevents binding of the Ad vectors to CAR (data not shown) and thus ablates the intrinsic specificity. This may be useful in vivo, in applications where CAR mediated uptake is undesired.

In summary, we have demonstrated the feasibility of the CAR.cRGD construct to target Ad-vectors with high efficiency to transformed endothelial cell lines as well as to primary endothelial and smooth muscle cells. It is conceivable that additional Ad resistant and

$\alpha_V\beta_{3/5}$ integrin expressing cell lines and tissues may become amenable to Ad infection via this strategy.

Methods

Cell Culture

Chinese Hamster Ovary (CHO) cells, H5V (mouse endothelial cell line derived from heart) and EOMA (mouse hemangioma-derived micro vascular cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL). Ramos cells (Burkitt lymphoma cells) and K-562 cells (chronic myelogenous leukemia cells from blast crisis) were cultured in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum, 100 units/ml Penicillin, 100 μg/ml Streptomycin and glutamax (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were a generous gift from E Pieterman (TNO Prevention and Health, Leiden The Netherlands) and were isolated as previously described [37,38] and grown in Medium 199 with 10% human serum. Mouse VSMC were isolated from aorta from male C57Bl6 mice as previously described [39] and cultured in DMEM with 10% newborn calf serum (NCS). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Production of recombinant Ad vectors

Recombinant E1, E3-deleted Ad-vectors expressing beta-galactosidase gene (Ad.LacZ) and firefly luciferase (Ad.Luc) under the control of the cytomegalovirus promoter (CMV) were kindly provided by respectively Dr. Willnow (Houston, USA) and Dr. Hoeben (LUMC, Leiden, The Netherlands). Recombinant adenovirus vector carrying the green fluorescent protein under control of CMV (Ad.GFP) was constructed using the Ad-Easy-1 system as previously described by [40]. Additionally, the virusses were propagated in PERC6 cells as described [41]. The purification process involved two rounds of CsCl ultra centrifugation and dialysis against dialysis buffer (25 mmol/l Tris, 137 mmol/l NaCl, 5 mmol/l KCl, 0.73 mmol/l NaH_2PO_4, 0.9 mmol/l $CaCl_2$, and 0.5 mmol/l $MgCl_2$, pH 7.45) followed by dialysis against the same buffer supplemented with sucrose (50 g/l). Plaque titration was performed on 911 cells according to standard techniques [42]**.** Aliquots of 50 μl virus were stored at -80°C. Generally, virus titers of the stocks varied from 1 x 10^{10} to 1 x 10^{11} pfu/ml.

Generation CAR-Avidin linker protein

The CAR-Avidin linker protein was generated by joining a series of PCR-generated fragments. In short, the extracellular domain of the Coxsackie Adenovirus Receptor (CAR) was obtained by PCR using the plasmid pCAR (kind gift of Prof. R. Hoeben, LUMC, Leiden) as template (oligo's: 5'-GCG GCC GCG GGT ACC CAC GGC ACG GCA G-3' and 5'-CTA GCT AGC AGC TTT ATT TGA AGG AGG GAC-3'). The avidin fragment was obtained by RT-PCR on total RNA from chicken fibroblasts with random hexamer oligonucleotides and subsequent PCR using primers 5'-CGC GGA TCC GCC AGA AAG TGC TCG CTG -3' and 5'- CCA TCG ATG GTC ACT CCT TCT GTG TGC G -3'. The CAR fragment was cloned into the pSG8 vector (generous gift of prof. Henk Stunnenberg, Nijmegen, the Netherlands), in front of the VSV and His6 tag. Avidin was cloned in frame into pSG8CAR behind the VSV and His6 tag. All constructs were sequence verified.

Production and purification CAR-Avidin linker protein

For production Cos-1 cells were transfected with pSG8CAR-Avidin using Fugene6 (Roche, Basel, Switzerland). Forty hours after transfection (serum-free, biotin-free culture medium), the supernatant, containing the linker proteins, was harvested. Linker proteins were purified from the supernatant by immobilized metal affinity chromatography using Talon metal affinity resin (Clontech, Palo Alto, USA). Equilibrated culture supernatant (300 mM NaCl, pH = 7.0 and 20% glycerol) was incubated with Talon, 20 minutes at room temperature. After extensive rinsing (50 mM $NaPO_4$, 300 mM NaCl, 20% glycerol, pH = 7.0), resin was pre-eluted (4 volumes; 50 mM $NaPO_4$, 300 mM NaCl, 2,5 mM imidazole, 20% glycerol) prior to its elution (10 volumes; 50 mM $NaPO_4$, 300 mM NaCl, 150 mM imidazole, 20% glycerol). Presence of linker protein in the purified samples was detected by SDS-PAGE and western blotting analysis using Hybond ECL nitro cellulose membranes (Amersham Biosciences, Buckinghamshire, UK) and antibodies P5D4 (α-VSV) or α-Avidin (Abcam, Cambridge, UK). Elution fractions 3 to 5 contained the linker protein and were dialyzed against PBS.

Quantification of CAR-Avidin linker protein

The linker protein was quantified by a biotin binding assay. 10 µl of the linker protein elution fraction or an avidin calibration range of 0.3 - 10 pM avidin was incubated with 0.2 µl ^{3}H-Biotin (Du Pont NEN Research Products, Boston, MA, USA) for 1 hour. The total reaction mixture was applied on a Sephadex G-50 column to separate CAR-Avidin bound biotin from the free biotin. The elution fractions were counted for ^{3}H-Biotin radioactivity using 5 ml of

Hionic fluor scintillation cocktail (Packard Instrument Co., Perkin Elmer, Boston, MA, USA) in a Packard 1500 TriCarb liquid scintillation analyzer. The summed radioactivity in peak fractions 3 to 5 correlated with the amount of avidin present in the sample (R^2= 0.997). Elution fraction 3, which had the highest concentrations, was used for experiments and stored at –80°C. A yield in the order of 900-1000 µg was typical.

Biotin binding assay

CAR-Avidin (5 µl of 30 nM) was incubated for 1hr at RT with bio-cRGD (cdFK(ε-C6-biotin)RGD), from Asynth Service BV (Roosendaal, Netherlands) at molar ratios ranging from 1:0.001 to 1:3, after which 2 µl of ^{3}H-Biotin (NEN) was added and the mixture was incubated again for 1h. To separate the CAR-Avidin-(^{3}H- or cRGD-) biotin bounded fractions from free ^{3}H-biotin the mixture was applied on a Sephadex G-50 column. ^{3}H-Biotin radioactivity in the elution fractions was measured after addition of 5 ml Hionic Fluor scintillation cocktail (Packard Instrument Co) in a Packard 1500 tricarb liquid scintillation analyzer. The summed radioactivity in peak fractions 4 to 6 corresponded to the ^{3}H-biotin binding capacity of the CAR-Avidin. This value was plotted for each sample containing different molar ratios of CAR-Avidin to bio-cRGD (1:0.001 to 1:3).

Infection assay

24 hours before infection, cells were seeded into 12 wells plates (Greiner). The CHO, H5V and HUVEC at 4.10^4, VSMC at 6.10^4, Ramos and K-562 at 1.10^5 and EOMA at $1,2.10^5$ cells per well. At the day of infection, three wells were trypsed to calculate the number of cells. After that, CAR-Avidin was incubated for 1 hour at RT with bio-cRGD in a total volume of 50 µl PBS. Then, the CAR-cRGD targeting construct was added and incubated for 1 hr with different amounts of Ad.Luc, Ad.GFP or Ad.LacZ. Subsequently 300 µl of cRGD-Ad diluted in PBS/2% horse serum was added to the cells. After 1 hr at 37°C, the media was changed and infection efficiency was determined 40 hrs after infection. Ramos and K-562 (suspension) cells were washed by centrifugation for 5 min at 1000 rpm in between the incubation steps.

Ad.luc infected cells were lysed in 300 µl reporter lysis buffer (Promega). Luc activity (Promega) and protein content (BCA assay, Pierce) was measured according to the protocol supplied by the manufacturer.

The Ad.LacZ infected cells were washed with PBS and fixed for 5 min at 4°C in 5.4% formaldehyde, 0.8% gluteraldehyde in PBS after which staining solution (5 mM potassium

ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM $MgCl_2$, 0.1% 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) in PBS) was added. After 4 hrs LacZ positive cells were visual and scored microscopically.

Ad.GFP infected cells were trypsinized gently, homogenized in PBS supplemented with 2% fetal calf serum and kept on ice until further analysis by flow cytometry (Becton – Dickinson). GFP fluorescence was detected at 530/30 nm FACscan (FL1 channel) following excitation with an argon ion laser source at 488nm. The forward-scatter/side-scatter plot was gated to exclude cellular debris from the analysis. The number of events/FL1 (which reflects the fluorescence intensity) was plotted against the total number of cells, and the percentage of GFP-positive cells was determined. For each sample, 10.000 events were collected.

Statistical analysis

Results are presented as mean ± SD values of three samples. The significance of differences between the experimental groups was calculated using a two-tailed Student's *t* test. The level of statistical significance of the difference was set at $P < 0.05$.

Acknowledgements

This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO and supported by grants from the Dutch Organization for Scientific Research (NWO 902-26-220), Dutch Heart Foundation (NHS 2001-141 and NHS 2003T201) and the Center of Medical Systems Biology (CMSB) established by the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NGI/NWO).

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6.

Targeting adenovirus vectors reduces liver tropism but does not enhance specific organ uptake.

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Abstract

Systemic administration of adenovirus (Ad) vectors results in gene delivery to the liver. To modify Ad vector tropism, we have generated a linker protein consisting of the virus binding domain of the coxsacky adenovirus receptor (CAR) genetically fused to avidin. In association with a biotinylated ligand, this CAR-Avidin linker protein can successfully retarget Ad vectors *in vitro*. Here, we set out to apply this targeting strategy *in vivo*. Two biotinylated peptide ligands were used to, respectively, target integrin $\alpha_V\beta_{3/5}$ expressing cells and lung endothelium. Systemic administration of both types of targeted Ad vectors resulted in an up to 85-fold reduced hepatic transgene expression. However, neither of the targeted Ad vectors resulted in increased transgene expression in the intended target tissue. Moreover, a substantial portion of the targeted Ad could not be recovered from any of the organs, indicative of efficient Ad neutralization. Indeed we observed that the maximum half-life of Ad in the circulation after systemic lactoferin treatment, which completely blocks hepatic Ad uptake was 8' as compared to 6' for untargeted Ad suggesting the presence of efficient extrahepatic elimination pathways. Apparently, rapid neutralization of targeted Ad in the circulation efficiently prevents uptake by target organs other than the liver.

Introduction

Adenovirus (Ad) mediated gene transfer is widely used as a powerful method to modulate gene expression *in vitro* and *in vivo* (reviewed by [1]) The vast majority of Ad used to date involves serotype 5 (Ad5). Application of Ad5 vectors is dependent on the expression of the cognate receptor, the Coxsackie Adenovirus Receptor (CAR) by the target cell [2,3]. Infection of CAR deficient cells, such as many tumours, endothelial and hematopoietic cells, with Ad vectors is very ineffective and can be achieved only at high multiplicities of infection. Thus efficient infection of these cells requires modulation of Ad tropism.

In vitro, several targeting approaches have been proven successful. In one of the strategies the capsid protein is genetic modified by inserting peptide ligands [4-22]. Another approach for targeting Ad vectors is based on conjugates. Here, the vector is equipped with a bifunctional adapter molecule able to bind the virus on the one hand and a marker protein on the target cell on the other hand [23]; (reviewed by [24]) [25-28]; (reviewed in [29]) The adapter can either associate with the native virus or with chemically or genetically modified capsid proteins. This targeting approach is more versatile than the genetic modification based

strategy, as it results in a flexible targeting system able to confer/ accommodate infection of a variety of cell types via the addition of different ligands.

Recently, we described the generation and *in vitro* characterization of linker protein CAR-Avidin for the targeting of Ad5 to alternative cell types. This linker protein consists of the virus-binding moiety of the endogenous receptor CAR, genetically fused to the biotin-binding moiety of avidin. Equipping CAR-Avidin with the oligodeoxy nucleotide ligand dA_6dG_{10} or the cyclic peptide ligand GRGDSP (cRGD) resulted in efficient targeting *in vitro* of both transformed and primary macrophages [30] and to both transformed and primary vascular smooth muscle and endothelial cells [31], respectively. The aim of the current study was to determine whether *in vivo* targeting of Ad vectors to extrahepatic tissue such as carotid artery or alveolar cells, can be effected via the CAR-Avidin linker protein.

Results

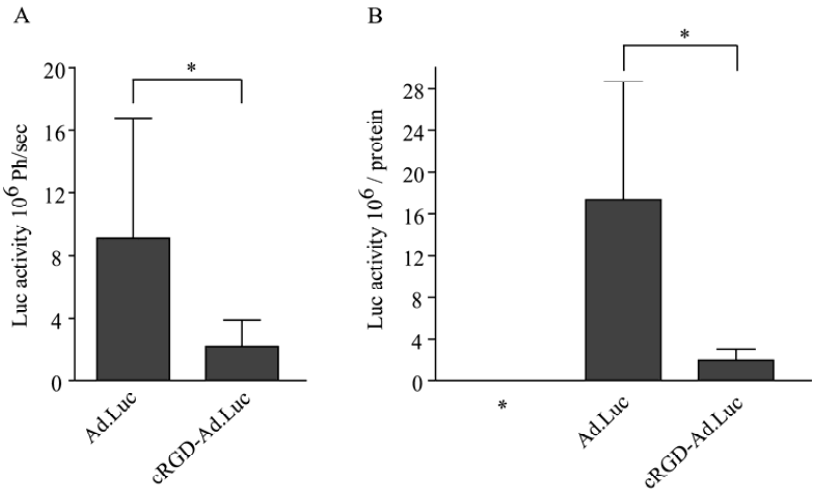

Figure 1. Effect of cRGD mediated targeting of adenovirus on luciferase expression in the liver. **(A)** Female C57Bl/6 mice received $2x10^9$ pfu Ad.Luc (i.p.). After 96 hours luciferin (150 mg/kg) was administered by i.p. injection and bioluminescent signals were recorded under full anesthesia for 1 minute. Data are presented as cumulated photon counts. **(B)** Liver lysates were prepared by homogenisation and subsequent freeze-thawing. Supernatants were used for determining luciferase activity using. Luciferase activity was corrected for protein concentration using BSA as standard. $P<0.05$ is indicated by an asterix.

Systemic administration of cRGD equiped Ad into mice.

To examine the capability of integrin targeted Ad vectors to mediate extrahepatic gene transfer and reduce the liver uptake normally seen after systemic application of Ad, either

untargeted Ad expressing luciferase (Ad.Luc) or Ad.Luc equipped with CAR-Avidin-cRGD (cRGD-Ad.Luc) was injected intravenously into mice. Two and four days after injection, *in vivo* gene transfer was monitored *in situ* via a high resolution CCD camera. Luciferase expression accumulated in time and was solely observed in the liver and not in other organs of both the Ad.Luc as well as cRGD-Ad.Luc treated mice. Compared to Ad.Luc treated mice, luciferase expression was decreased 3-fold in mice that had received cRGD-Ad.Luc (Fig. 1A). Because layers of tissue may limit photon emission from inner organs, luciferase activity was also measured in liver lysates. These data confirmed that the liver had indeed been infected and that transgene expression by liver was considerably reduced after cRGD targeting (11.5 fold; $P<0.01$) (Fig 1B).

To determine whether the decreased luc activity after targeting was caused by a reduction in the percentage of infected cells and not only by a reduction of virus particles entering a cell, untargeted Ad expressing β-galactosidase (Ad.lacZ) or targeted Ad.LacZ (cRGD-Ad.LacZ) was injected systemically. Four days after intravenous administration, β-galactosidase staining of the livers of mice that had received cRGD-Ad.LacZ revealed only 1% $LacZ^+$ cells. In comparison, approximately 80% of hepatic cells stained positive for β-galactosidase Fig. 2). In addition, with cRGD targeted Ad the cellular staining intensity seemed to be quenched as compared to untargeted Ad (Fig. 2).

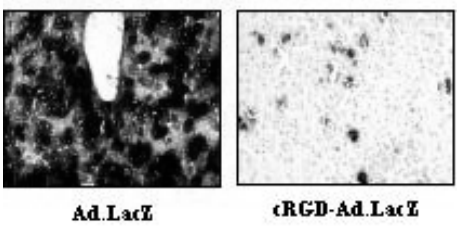

Figure 2. Effect of cRGD mediated targeting of adenovirus on ß-galactosidase expression in the liver. Female C57Bl/6 mice received cRGD-Ad.LacZ or untargeted Ad.LacZ ($1x10^9$ pfu; i.v. injection). Five days after infection mice were sacrificed, livers were excised and cryosections were made and stained for ß-galactosidase or with hematoxylin/ eosin.

Administration of cRGD equipped Ad to mice with carotid artery injury.

To determine whether the endothelium constituted a barrier to infection, a carotid artery segment was injured by guide wiring prior to systemic virus administration. The guide wire injury will result in activation of flanking endothelial cells and medial vascular smooth muscle cells (VSMC) and subsequently in an increase in $\alpha_V\beta_{3/5}$ integrin expression. Systemic administration of cRGD-Ad.LacZ 1 or 5 hours after denudation of the internal carotid artery did not result in an increased amount of $LacZ^+$ cells in the vessel wall (data not shown). Because the anatomical position of the carotid artery could be incompatible with the

dynamics of Ad infection, we also administrated Ad focally by instillation in an uninjured or a denuded carotid artery segment. Similar to above, cRGD equipped Ad vectors did not enhance transgene expression in intact endothelium nor in denudated vessels (Fig 3).

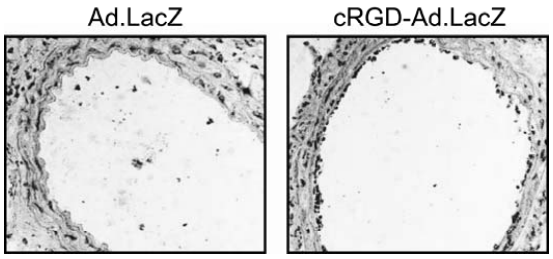

Figure 3. Effect of cRGD mediated targeting of adenovirus on LacZ expression in the vessel wall. The right common carotid artery of ApoE-/- mice was denuded by 3 rotational passes of a 0.36 mm guide wire. Subsequently, Ad (1.5×10^9 pfu) was instilled into the denuded common carotid artery segment via the external carotid artery after prior ligation of the common carotid artery proximal and distal to the bifurcation point. The Ad was left in situ for 15' and removed. Five days after infection mice were sacrificed, tissues were isolated and cryosections of the common carotid arteries were stained for ß-galactosidase or with hematoxylin/ eosin.

Lung specific targeting of Ad vectors.

To determine whether Ad retargeting would be successful with an alternative ligand for a more accessible organ, we have explored the potential of a lung specific peptide, GFE1 [32], in CAR-Avidin aided gene transfer in vivo. First, biodistribution to the lung of this peptide was confirmed by systemic administration of biotinylated GFE1 coupled to ^{125}I labelled avidin. Figure 4 shows the specific organ uptake of the avidin$^{125\text{-}I}$ bound peptide 1 hour after systemic injection. Biotin was included as a negative control. As expected and

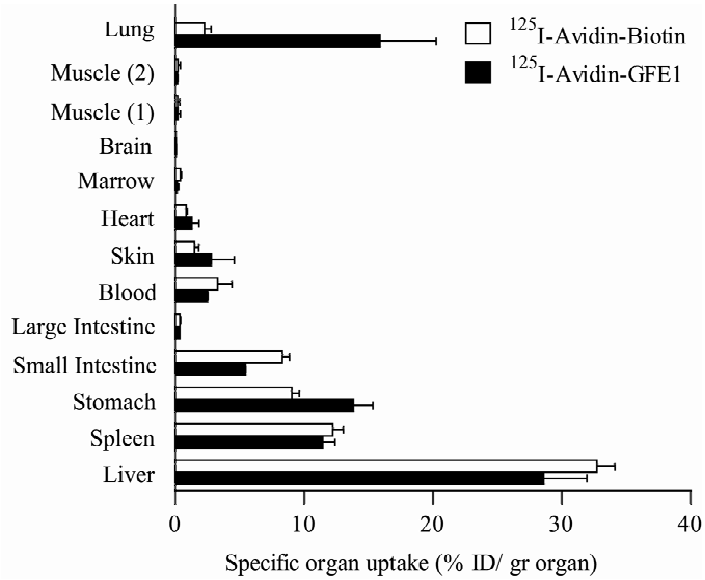

Figure 4. Biodistribution of ^{125}I-Avidin-GFE1 after intravenous injection into mice. ^{125}I-avidin-biotin or ^{125}I-avidin-GFE1 (molar ratio of 1: 1; 156749 dpm, in 100 µl PBS) was injected intravenously into female C57Bl/6 mice. Tissue distribution was determined 1h after injection. Tissue accumulation is expressed as % of the injected dose per gram wet tissue and was corrected for radioactivity associated with tissue-entrapped plasma.

already reported by Trepel et al., avidin bound GFE1 showed a much higher lung uptake than the biotin control (7-fold increase). Second, Ad.Luc was equipped with GFE1 (GFE1-Ad.Luc) for in vivo application. Biotin saturated CAR-Avidin (biotin-Ad.Luc) and untargeted Ad.Luc served as control. Five days after systemic injection, luciferase activity was determined in different organs. In comparison to Ad.Luc administration, the mice treated with biotin-Ad.Luc and GFE1-Ad.Luc displayed an 85- and 19- fold reduction in hepatic luciferase activity, respectively (Fig. 5A). Figure 5B shows the overall organ distribution of the luciferase expression after administration of GFE1-Ad.Luc, biotin-Ad.Luc or Ad.Luc. In most tissues, including lung, luciferase expression was found to be lower in the GFE1-Ad.Luc than when in Ad.Luc treated mice. Thus, similar to the results obtained with the cRGD ligand, the GFE1 peptide did not enhance specific organ uptake.

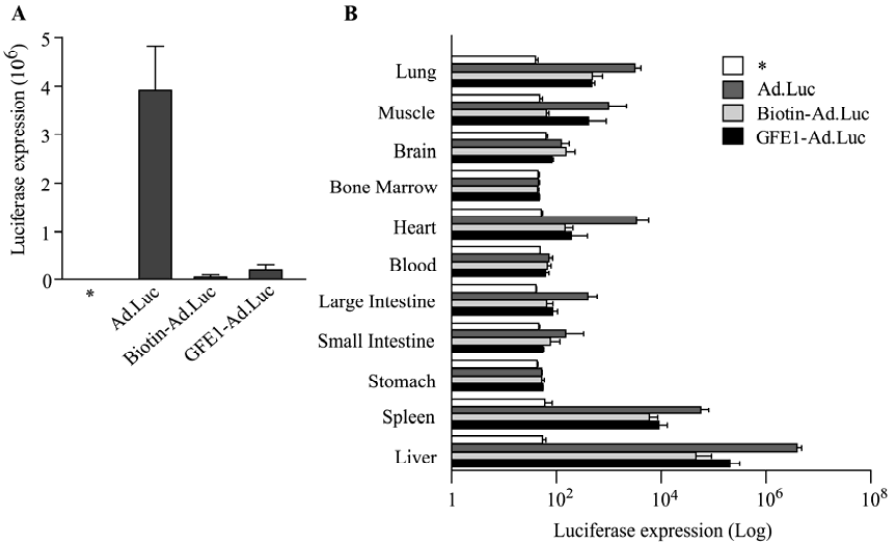

Figure 5. Effect of GFE1 mediated targeting of adenovirus on biodistribution luciferase expression. **(A)** untargeted Ad.Luc, biotin-Ad.Luc or GFE1-Ad.Luc were systemically administered to female C57Bl/6. Liver lysates were prepared 120 hours after infection by homogenisation and subsequent freeze-thawing. Supernatants were used for determining luciferase activity. **(B)** The organ distribution profile of luciferase expression 120h after i.v. administration of GFE1-Ad.Luc, biotin-Ad.Luc, untargeted Ad.Luc or buffer (uninfected) to female C57Bl/6 mice is plotted. Mind the logarithmic X-axis.

Determining Ad stability *in vivo*.

As the previous experiments made clear, a substantial portion of Ad could not be recovered from any of the organs, therefore *in vivo* kinetics studies were performed.

Untargeted Ad.Luc had a half-life of approximately 6 minutes *in vivo* (fig. 6A) and was efficiently cleared by the liver resulting in efficient infection. When animals were pre-treated systemically with lactoferrin, hepatic uptake of Ad virus particles was nearly completely blocked (fig. 6B). Under these conditions however, half-life of untargeted virus was only increased to 8 minutes (fig. 6A). This relatively minor increase in half-life indicates that neutralization of virus in the systemic circulation is extremely fast.

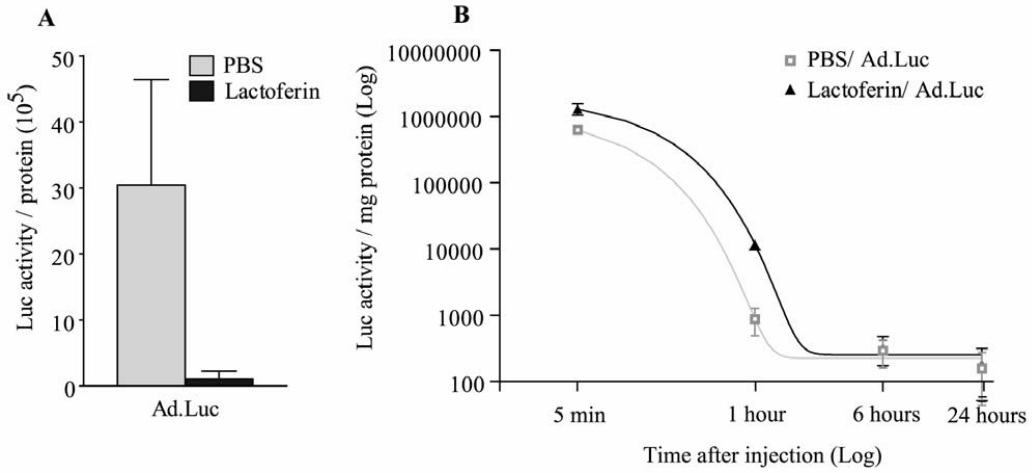

Figure 6. Effect of systemic lactoferrin treatment on Ad half-life after i.v. administration. (A) Mice received bovine lactoferin (i.v., 70mg/kg) (grey line, triangles) or PBS (black line, squares) 2 minutes before Ad.Luc ($1{,}5.10^9$ pfu) administration. Presence of circulating infectious particles was determined by blood sampling and subsequent incubation of the samples on AT3 cells. Luciferase activity in AT3 cells is corrected for protein concentration and plotted against the time of blood sampling. **(B)** Luciferase activity in livers of mice determined 5 days after lactoferrin and Ad.Luc administration. Luciferase activity was determined as previously mentioned.

Discussion

In this paper we report our efforts to target adenovirus vectors to alternative cell types *in vivo*. Ad linked to either the integrin binding peptide cRGD [33,34] or the lung specific peptide GFE1 [32] via CAR-Avidin was able to reduce liver uptake after systemic administration. While cRGD-Ad has already been shown to be effective in delivering genes to vascular cells *in vitro* [14,35] and GFE1 was demonstrated to be lung specific *in vivo*, neither cRGD-Ad nor GFE1-Ad was able to increase transgene expression by respective target tissue *in vivo*. In addition we demonstrate that the half-life of adenovirus in blood is rather short even after ablation of liver uptake by lactoferrin suggesting that other elimination pathways are functional in the clearance of adenovirus. This could contribute to the apparent failure of efficient target organ uptake *in vivo*.

Because of the clinical relevance, efficient *in vivo* targeting – in other words enhanced target organ uptake and quenching of the intrinsic tropism - of Ad vectors is highly desired. Though attempted extensively, successful targeting of Ad *in vivo* is limited to local [36-38] or intra-organ injections [39] of the virus. To our knowledge, increased target organ uptake of Ad after systemic injection has only been shown by Izumi and colleagues [40], who administered engineered Ad.luc containing CD40 on their fiber proteins in transgenic mice with lung vasculature specific CD40 expression. The detour Izumi took to accomplish retargeting demonstrates the difficulty in retargeting Ad in vivo.

In the current paper we succeeded in efficiently reducing liver uptake by applying the CAR-Avidin linker protein equipped with ligands for alternative receptors to Ad. Apparently, the CAR-Avidin linker protein is capabable of blocking the interaction of Ad fiber knob with its natural receptor, CAR. As both untargeted Ad and cRGD-Ad were from the same batch, we can exclude that batch-related factors are underlying the observed phenomena. Parallel in vitro studies confirmed that the cRGD Ad complexes were still functional, as they significantly enhanced gene transfer to vascular cells *in vitro*. Moreover, electron microscopy studies revealed that cRGD equipped Ad did not form large aggregates >100-150 nm, that are unable to penetrate the fenestrae in the liver (data not shown). Thus, a major requirement for successful retargeting of Ad vectors to specific cell and tissue targets has been achieved.

To redirect Ad vectors we have linked cRGD to Ad vectors via the CAR-Avidin linker protein. This peptide was shown to display a high affinity for $\alpha_V\beta_{3/5}$ integrins expressed on activated (angiogenic) endothelial cells and has been widely exploited in for targeting strategies of Ad [33,34] and other drug carriers i.e. liposomes [41]. In the current study, cRGD equipped Ad vectors have been used to target mechanically injured carotid arteries. Unfortunately, cRGD-Ad failed to infect both quiescent endothelial cells as well as activated endothelial cells flanking the site of injury after systemic administration *in vivo*. Furthermore, medial vascular smooth muscle cells at the site of injury were not infected as well. Since erythrocytes ubiquitously express integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ it is conceivable that the cRGD ligand is an inappropriate targeting molecule for systemic application, and this could explain our negative results. This is in line with findings of Haubner and colleagues [42], who have investigated several RGD based compounds. Interestingly, we were unable to detect any infection of vascular cells by cRGD-Ad even after local incubation in the absence of erythrocytes, suggesting that erythrocyte scavenging cannot be held accountable for the lack of vascular targeting by Ad in vivo. Several studies have demonstrated enhanced $\alpha_V\beta_{3/5}$ expression on endothelial and vascular smooth muscle cells after injury [43-45]. However,

$\alpha_V\beta_{3/5}$ mediated uptake is relatively slow and we cannot exclude the possibility that the time frame of upregulation of $\alpha_V\beta_{3/5}$ expression is incompatible with the currently applied infection protocol.

To avoid the possibility of erythrocyte mediated sequestration of cRGD-Ad or poor transendothelial permeation, a lung specific targeting moiety was used. The lung is a relatively large organ with high blood flow and thus easily accessible. Our targeting moiety was proven to be effective, since iodinated avidin-bioGFE1 was specifically taken up by lung. Nevertheless, the GFE1-Ad did not enhance transgene expression in the lung. It is possible that the in vivo stability of the ad vector and/or the local lung-specific blood flow conditions are incompatible with the attachment and uptake of the virus particles by the target cells.

Completely blocking liver uptake with lactoferin had no dramatic effect on the half-life of the untargeted virus (only a 33% increase). Due to the high blood flow through the liver (24% of the cardiac output) the slightly prolonged retention in the blood is probably not sufficient for the aimed target organs to take up the virus. Upon inhibition of liver uptake, Ad is rapidly neutralized by erythrocytes through binding of RGD motives to αvβ3/ αvβ5. This compromises virus stability after intravenous administration. So any retargeting approach will have to compete with systemic neutralization, and apparently the two ligands we have selected are not capable of doing this.

In conclusion CAR-Avidin has shown to efficiently detarget Ad from the liver upon equipping the Ad with novel ligands. This decrease in liver tropism however, was not accompanied by an increased transgene expression in novel target cells.

Methods

Cells

Cos-1, H5V and EOMA cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 100 units/ ml Penicillin (Invitrogen), 100 µg/ml Streptomycin (Invitrogen) and glutamax (Invitrogen). Mouse VSMC were isolated from aorta from male C57Bl6 mice as previously described [46] and cultured in DMEM with 10% newborn calf serum (NCS). One day prior to transfection Cos-1 cells were detached from plastic with 1% Trypsine/ 10 mM EDTA in PBS and seeded to 50% confluency. Cells were cultured in a humidified atmosphere of 5% CO_2

Production of recombinant Ad vectors

Recombinant E1, E3-deleted Ad-vectors expressing beta-galactosidase gene (Ad.LacZ) and firefly luciferase (Ad.Luc) under the control of the cytomegalovirus promoter (CMV) were kindly provided by respectively Dr. Willnow (Houston, USA) and Dr. Hoeben (LUMC, Leiden, The Netherlands). Additionally, the Ad vectors were propagated in PERC6 cells as described [47]. The purification process involved two rounds of CsCl ultra centrifugation and dialysis against dialysis buffer (25 mmol/l Tris, 137 mmol/l NaCl, 5 mmol/l KCl, 0.73 mmol/l NaH_2PO_4, 0.9 mmol/l $CaCl_2$, and 0.5 mmol/l $MgCl_2$, pH 7.45) followed by dialysis against the same buffer supplemented with sucrose (50 g/l). Plaque titration was performed on 911 cells according to standard techniques [48]. Aliquots of 50 µl virus were stored at -80°C. Generally, virus titers of the stocks varied from 1 x 10^{10} to 1 x 10^{11} plaque forming units per ml (pfu/ml).

Production, purification and characterization of the linker protein

The CAR-Avidin linker protein was produced, purified and characterized as previously described [30]. In short: CAR-Avidin was produced by transient transfection of Cos-1 cells with pSG8CAR-Avidin using Fugene6 (Roche, Basel, Switzerland) under serum free conditions. Thirty two hours after transfection the linker protein was harvested and purified from the supernatant by immobilized metal affinity chromatography using Talon metal affinity resin (Clontech, Palo Alto, USA). Culture supernatant was equilibrated (addition of 5M NaCl to an end concentration of 300 mM NaCl, pH was adjusted to 7.00 using 50% HCl in PBS, 100% glycerol was added to an end concentration of and 20% glycerol) and incubated for 20 minutes at room temperature with Talon. After extensive rinsing with buffer (50 mM $NaPO_4$, 300 mM NaCl, 20% glycerol, pH = 7.0), washing with 4 volumes pre-elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 2,5 mM imidazole, 20% glycerol) the resin was eluted with 10 volumes elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, 20% glycerol). SDS-PAGE and western blotting analysis of all purification steps and elution fractions showed that the linker protein was only present in elution fractions 3 to 5 that were subsequently extensively dialyzed against PBS and used in all experiments. The concentration of the active component was determined in the biotin binding assay as previously described using avidin (Sigma Aldrich, St Louis, MO, USA) as standard.

Ligands

The lung specific GFE1 peptide (CGFECVRQCPERC;[32]) was synthesized as N-terminally biotinylated peptide by standard Fmoc based solid-phase chemistry and provided by J.W. Drijfhout (LUMC, Leiden, The Netherlands). The quality was checked by high resolution LC-MS mass spectroscopy. The bio-cRGD (cdFK(e-C6-biotin)RGD) was obtained from Asynth Service BV (Roosendaal, Netherlands),

Targeting conditions

CAR-Avidin and peptide ligands were incubated at a 1: 1 molar ratio for 1 hour at room temperature, as was determined in previous experiments [30,31]). Complex formation of adenovirus and CAR-Avidin-*ligand* was facilitated by incubating CAR-Avidin-*ligand* at a concentration of 50 nM with the appropriate amount of virus for 1 hour at room temperature.

Bioluminescent reporter imaging

12 wks old female C57Bl/6JIco mice (Charles river, The Netherlands), fed standard chow diet (Hope Farms, Woerden, NL) *ad libitum,* were injected with Ad.Luc ($2x10^9$ pfu). Bioluminescent signals were determined 4 days after Ad injections using the Xenogen IVIS imaging system (IVIS 100). Approximately 5 minutes before imaging the living mice were injected luciferin, (150 mg/kg) intraperitoneally (ip). The mice were anaesthetized with isofluorane/oxygen and placed on the imaging stage. Total photon emission of each animal was acquired for 1 minute. Captured images were quantified using the Living Image software (Xenogen Corp, Almeda, CA) and the IGOR software (WaveMetrics Corp, Lake Oswego, OR). Bioluminiscence from the region of interest was expressed via a pseudo color scale (Red most intense and Blue least intense luminescence) and data were presented as the cumulative photon counts collected within each region of interest. Because layers of tissue may limit photon emission from inner organs, 4 days after Ad injection the livers of mice were dissected to verify the results from the bioluminescent reporter imaging experiment by determination of the luciferase activity in liver lysates

Luciferase enzymatic assay

The liver extracts were prepared by homogenisation with the minibead beater in reporter lysis buffer (Promega), two cycles of freeze-thawing and 2 min. of centrifugation at maximum speed. Supernatants were used for determining protein-normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of liver extract in a monolight luminometer

(BD Biosciences). Protein content was measured in a 96-well microtiter plate using the BCA protein assay kit (Pierce). Absorbance at 562 nm was determined in a microplate reader.

Local gene transfer

9-10 weeks old ApoE-/- mice, fed regular chow diet *ad libitum*, were used for the local gene transfer studies. Local gene transfer was ensured using a procedure developed by Von der Thüsen [49] In short: with use of a midline neck incision, the left external carotid artery was looped proximally and tied off distally with 6-0 silk suture (Ethicon). Additional 6-0 silk ties were looped round the common and internal carotid arteries for temporary vascular control during the procedure. A transverse arteriotomy was made in the left external carotid artery, and a 0.36-mm flexible angioplasty guidewire was advanced by 1 cm via a transverse arteriotomy of the external carotid artery, and endothelial denudation of the common carotid artery was achieved by 3 rotational passes. In one experiment, the animals were inoculated i.v. with 1,5 $\times 10^9$ pfu of Ad.LacZ or cRGD-Ad.lacZ in 200 µl of phosphate buffered saline one and five hours after denudation. In a second experiment, immediately after angioplasty, 10 µl of adenoviral suspension (1.5×10^9 pfu/ml) was instilled into the right common carotid artery via the external carotid. The suspension was left *in situ* for 10 min and was subsequently drawn off before ligation of the external carotid and closure of the skin wound with silk sutures.

Tissue harvesting and histological analysis

Five days after Ad incubations, carotid artery specimens were obtained and transverse 5 µm cryosections prepared after in situ perfusion fixation with formalin as described [50]. Cryosections were routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica, Darmstadt, Germany). ß-Galactosidase was demonstrated by incubation with staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM $MgCl_2$, 0.1% 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) in PBS) at 37°C O/N. Sections were stained immuno histochemically with antibodies against α-SM-actin (clone 1A4; diluted 1:500; Sigma) and CD31(rat anti-mouse, BD pharmingen; diluted 1:200). To detect specific Ab binding goat anti-mouse IgG peroxidase conjugate (dilution 1:100; Nordic, Tilburg, the Netherlands) was used as secondary antibodies, with 3,3'-diamino-benzidine, nitro blue tetrazolium as enzyme substrates (all Sigma) and for CD31 the ABC-AP kit and Vector-Red Substrate was used (Vector laboratories).

Biodistribution of GFE1

Recombinant avidin (SIGMA, St. Louis, USA) was radioiodinated at pH 10.0 with carrier free ^{125}I according to a modification [51] of the ICI method [52]. Free ^{125}I was removed by Sephadex G-25 gel filtration. GFE1 was incubated with ^{125}I- Avidin at a molar ratio of 1: 1. For the *in vivo* bio-distribution experiments, 10-12-wk-old female C57Bl/6 mice of weight 22-24 g from Broekman Instituut BV (Someren, The Netherlands) were used and fed *ad libitum* with regular chow diet. Mice were anaesthetized by subcutaneous injection of ketamine (75 mg/kg, Eurovet), droperidol (1 mg/kg), fluanisone (0.75 mg/kg), and fentanyl (0.04 mg/kg) (all from Janssen-Cilag, Beerse Belgium). Mice were injected with indicated ligand via the tail vein. One hour after injection the experiment was terminated, organs were removed and the organ bound radioactivity determined.

Bio-distribution of targeted Ad

For the *in vivo* virus bio-distribution experiments, 10-12-wk-old female C57Bl/6 mice of weight 22-24 g from Broekman Instituut BV (Someren, The Netherlands) were used and fed *ad libitum* with regular chow diet. On day 0, mice were injected with $1*10^9$ pfu of the appropriately targeted Ad.Luc or 100µl PBS in case of the uninfected control. Five days after injection, the experiment was terminated, the organs were removed and snap frozen in liquid nitrogen. Proteins were isolated after homogenisation of organ samples and subsequent solubilization in 1* reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined according to the protocol supplied by the manufacturer.

Decay study using lactoferrin

16-17 weeks old C57Bl/6JIco mice (Charles river, The Netherlands) were injected intravenously either with dissolvent or bovine lactoferrine (Serva, Brunschwig Chemie), 70mg/kg at t = -2 min. At t = 0 min, both groups of mice received $1,5.10^9$ pfu/mice Ad.Luc intravenously. Blood samples were taken by tail bleeding at 5 min, 60 min, 6 hours and 24 hours. At day 5 liver, heart, spleen and lung were isolated. To assess the presence of circulating infectious particles, AT3 cells were incubated for 1 hour with the blood samples. After 24 hours, protein extracts were prepared by addition of reporter lyses buffer (Promega) to the AT3 cells and two cycles of freeze-thawing followed by 2 min. of centrifugation at maximum speed. Supernatants were used for determining protein-normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of AT3 extract in a monolight luminometer (BD Biosciences). Protein content was measured in a 96-well microtiter plate

using the BCA protein assay kit (Pierce). Absorbance at 562 nm was determined in a microplate reader. $T_{½}$ were calculated from the luciferase activity at different timepoints using Graphpad Prism, software and a one-phase exponential decay model.

Statistics

Experiments were performed in triplicate and presented as mean ± standard deviation (s.d.). P-values were calculated by a two-tailed unpaired student's T-test. Data were considered to be significantly different when $P< 0.05$, indicated with an asterix (*) in the figures.

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7.

Reduced estrogen receptor alpha levels do not limit the anti-inflammatory effects of 17-beta-estradiol in endothelial cells

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Abstract

Objective: In the present study, the role of estrogen receptor alpha (ERα) in the anti-inflammatory effect of 17-β-estradiol (E_2) has been examined. ***Method:*** Endothelial cell lines with reduced ERα levels were generated by transduction with lentiviral vectors expressing short hairpin (sh)RNA constructs against ERα (shERα). Real time PCR was performed to quantify the expression levels of inflammatory cell adhesion molecules in stably transduced endothelial cells. ***Results:*** Expression levels of the adhesion molecules, E-selectin and intercellular adhesion molecule-1 (ICAM-1) were significantly induced by TNFα treatment, and were significantly inhibited by pre-treatment with E_2. Surprisingly, the shERα expressing endothelial cells, which displayed 50% reduced ERα mRNA levels and activity, responded in an identical manner to TNFα plus and minus E_2 pre-treatment. Complete abrogation of ERα activity, by supplementation of the antagonist ICI, however, did block the E_2 effect. ***Conclusion:*** ERα activity is required for the anti-inflammatory effect of E_2 but not in a "rate-limiting mode"

Introduction

Atherosclerosis is considered to be a chronic inflammatory process. One of the initial events involves the recruitment of inflammatory cells from the circulation into the developing lesion. This process is dependent on the expression of adhesion molecules such as E-selectin, vascular cellular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Expression of adhesion molecules is increased in atherosclerotic lesions [1-5], whereas deficiency of these molecules has resulted in a reduction of atherosclerostic lesion size and number [6-9].

Atheroprotective properties of E_2 have been demonstrated in several animal models [10-12], however, the underlying mechanisms remain obscure. Adhesion molecules constitute a possible target for E_2, even though experimental and epidemiological studies have reported conflicting results. Some have reported that hormone replacement therapy in post-menopausal women with coronary artery disease results in a reduction of soluble adhesion molecules [13-15], while other studies did not report significant changes [16-18]. Also, in vitro studies, which measured the effect of E_2 on endothelial expression of adhesion molecules have reported both enhanced [19,20] and reduced [21-23] expression level of adhesion molecules.

The actions of E_2 are mainly exerted via estrogen receptors (ERs), which classically serve as ligand-activated transcription factors. To date two ERs, ERα and ERβ have been

identified [24-26]. Both ERα and ERβ are present in vascular endothelium [27], but their physiological roles are incompletely understood. In vitro studies indicate that both ERα and ERβ can mediate the anti-inflammatory effect of E_2 with respect to the expression of adhesion molecules [28,29]. Interestingly, ERα levels in atherosclerotic vessels have been documented to be lower as compared to their levels in normal vessels and vessels with a mild degree of atherosclerosis [30-32]. Whether the reduced level of functional ERα in endothelial cells results in a reduced response to E_2 and thus aggravates the atherosclerotic process is not known.

In the present study, we have evaluated the effect of E_2 on TNFα-induced expression of adhesion molecules in a mouse endothelial cell line. To gain insight into the biological role of ERα and the importance of ERα level in endothelial function, lentiviral vectors expressing short hairpin (sh)RNA targeted to ERα (shERα) were designed. Silencing of ERα gene expression as well as ERα functioning was established. However, in contrast to total ablation of ERα activity, 50% reduction in ERα activity did not affect the E_2 signaling cascade regarding down-regulation of TNFα-induced expression of adhesion molecules. Thus, in the current study we found that ERα is required but their levels are not rate-limiting in the anti-inflammatory response of E_2.

Results

Expression levels of adhesion molecules in mouse endothelial cells

Expression levels of several adhesion molecules were determined in a mouse endothelial cell line (H5V), both under basal as well as under stimulatory conditions. Whereas VCAM-1 levels were undetectable, ICAM-1 and E-selectin levels were expressed under basal conditions, with E-selectin showing the most abundant levels. TNFα treatment dose dependently induced the expression of ICAM-1, which leveled off at a dose of 100 units TNFα per well (Figure 1A).

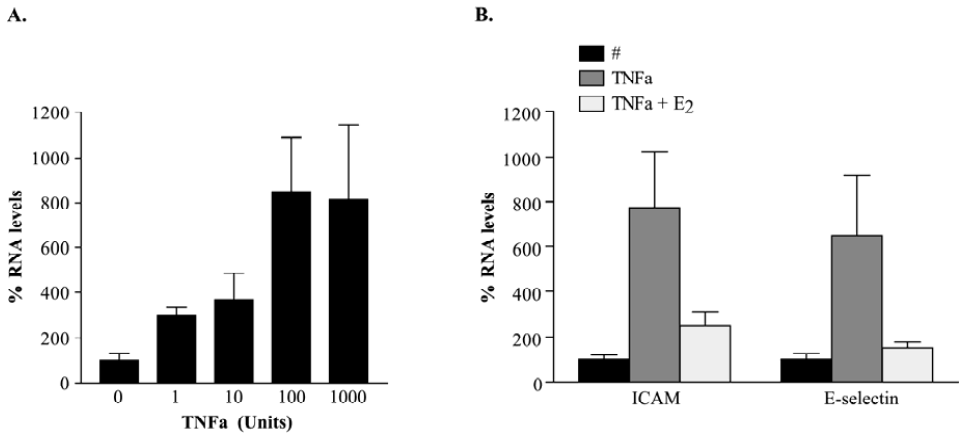

Figure 1. Expression of adhesion molecules in endothelial cells
(A) Endothelial cells were incubated with the indicated doses range of TNFα. Five hours after treatment RNA was extracted and subjected to taqman analysis to measure ICAM-1 levels. (B) The effect of E_2 on ICAM-1 and E-selectin expression in TNFα stimulated endothelial cells was assessed by taqman analysis. Dissolvent or 10^{-6}M E_2 was added 19 hours prior to TNFα (100 Units) treatment. The ratio of ICAM-1 / HPRT and E-selectin / HPRT of untreated cells was arbitrarily set as 100 for control. Data represented as mean ± SD.

E_2 and TNFα induced expression of adhesion molecules

As depicted in figure 1B, pretreatment of H5V cells with E_2 (10^{-8}M, 24 hours) significantly diminished the TNFα-mediated increase in both ICAM-1 and E-selectin levels. ICAM-1 levels were reduced from 774 ± 248% to 246 ± 61% and E-selectin levels from 650 ± 270% to 153 ± 24% ($P<0.05$). In a another endothelial cell line, hemangioendothelioma-derived cells (EOMAs), we were able to reproduce the repressive effect of E_2 (data not shown).

ERα levels and activity in shERα expressing endothelial cells

To knockdown the endogenously expressed ERα, a near 100% stable shERα expressing endothelial cell line was generated by selecting for GFP positive cells. RNA was isolated from Lenti-Empty (control) and Lenti-shERα cells 24 hours after E_2 (10^{-8}M) treatment to evaluate the silencing effect. As depicted in Fig 2A, real-time PCR analysis demonstrated up to 50% reduced ERα RNA levels in the shERα expressing H5V cells. In control as well as shERα expressing endothelial cells, ERα RNA levels were not modified upon E_2 and ICI treatment (data not shown)

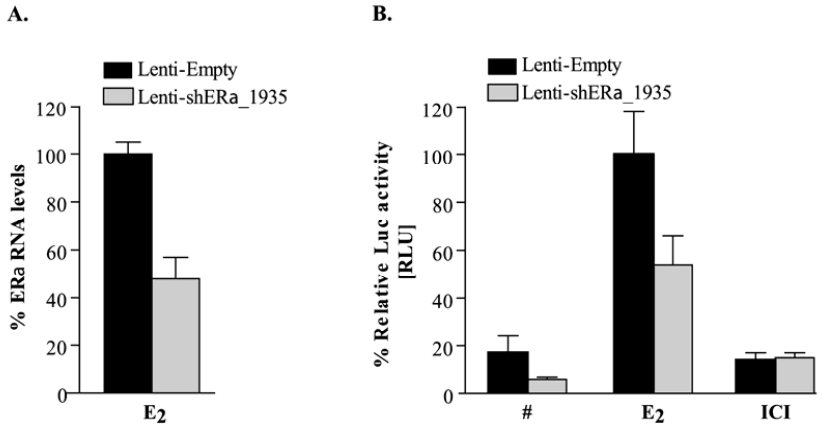

Figure 2. Mouse ERα mRNA and activity in stable shERα expressing endothelial cells
(A) RNA was extracted from H5V cells infected either with Lenti_Empty or Lenti_shERα constructs. ERα levels were assessed by taqman analysis. HPRT was used as internal standard. Data represented as mean ± SD. (B) Both Lenti_Empty and Lenti_shERα transduced H5V cells were co-transfected with pERE-Luc and pCMV-LacZ. The cells were stimulated with 10^{-8}M E_2 or 10^{-8}M E_2 plus 10^{-6}M ICI for 24 hours. Luciferase activity was measured 48 hours after transfection. Data represented as mean ± SD. E_2 induced luciferase activity in Lenti_Empty cells was arbitrarily set as 100%.

Subsequently, the effect of reduced ERα levels on ERα mediated transcription was evaluated. To this end, cells were transfected with a reporter plasmid carrying the estrogen response element upstream of the luciferase gene (pERE-Luc) and were treated with either dissolvent, E_2 or E_2 + ICI. As a result, control H5V cells showed enhanced ERα activity upon E_2 treatment, which was totally abolish by ICI treatment (Fig 2B). On the other hand, introduction of shERα led to a significant repression of ERα activity, as reflected by a 64% reduction under basal conditions and 46% reduction in the E_2 treated cells. Thus, shERα expressing lentiviral vectors significantly reduced ERα RNA levels and suppressed ERα mediated transcription.

ERα Knockdown in H5V cells and response to TNFα and E_2

The ERα knockdown H5V cells were used to explore the role of ERα in the E_2-induced repression of E-selectin and ICAM-1 expression. As shown in Fig 3, also in the ERα knock-down cell line, TNFα induces the expression of E-selectin and ICAM-1 levels. Though, reduced ERα levels, E_2 was able to significantly reduce the TNFα induced effect. On the

other hand, the antagonist ICI, which has been shown to silence ERα activity completely (Fig 2B), did abrogate the E_2 effect on the expression of adhesion molecules (Fig 3). Thus, while complete abolishment of ERα activity did abort the repressive effect of E_2 on adhesion molecule, reduced ERα activity did not.

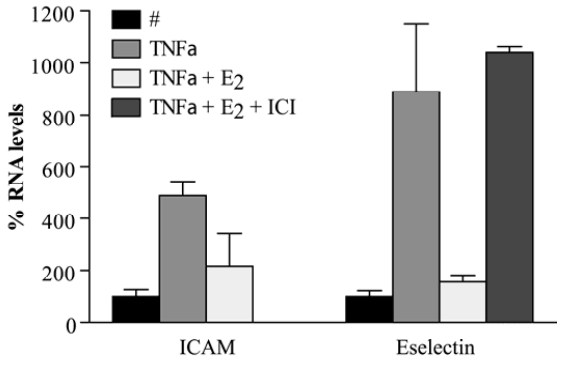

Figure 3. Expression of adhesion molecules in shERα expressing endothelial cells
Either dissolvent, 10^{-6}M E_2 or 10^{-6}M E_2 plus 10^{-6}M ICI was added 19 hours prior to TNFα (100 Units) treatment in Lenti_shERα transduced endothelial cells. Five hours after treatment RNA was extracted and subjected to taqman analysis to measure ICAM-1 levels and E-selectin levels. The ratio of ICAM-1 / HPRT and E-selectin / HPRT of untreated cells was arbitrarily set as 100 for control. Data represented as mean ± SD.

Discussion

Our results demonstrate that E_2 inhibits TNFα-induced expression of ICAM-1 and E-selectin levels in endothelial cells. To determine the role of ERα activity in this process, a stable shERα expressing endothelial cell line was generated. This shERα expressing cell line contained 50% reduced ERα mRNA levels resulting in 50% decreased ERα activity. Repression of endogenously expressed ERα, however, did not affect the E_2 inhibitory effect on expression of endothelial adhesion molecules. In contrast, complete silencing of ERα activity by use of the antagonist ICI did efficiently reverse the E_2 effect. Apparently, ERα activity is required for the anti-inflammatory response of E_2 with regard to inhibition of adhesion molecules, but the number of ERα molecules and level of ERα activity does not limit this response.

The observation that pre-treatment with E_2 significantly reduces the cytokine-induced expression of the endothelial adhesion molecules E-selectin and ICAM-1, implicates that E_2 makes the endothelium less responsive to the inflammatory microenvironment. In vitro studies with opposite results have also been published [19,20]. In those studies, E_2 was added simultaneously with the cytokine instead of before treatment as applied here, which could explain the discrepancy. Remarkably, in animal models beneficial effects were only observed

if E_2 was administrated prior to the development of atherosclerosis and not when arterial damage was present prior to hormone treatment [33-36]. From these studies we hypothesize that E_2 prevents atherosclerosis by interfering either prior to injury or very early post-injury.

RT-PCR analysis revealed that the TNFα-induced mRNA expression levels of E-selectin and ICAM-1 are reduced by E_2, indicating that the down regulation occurs at the transcriptional level. Since the ER antagonist ICI blocks the inhibitory effect of E_2 and the endothelial cells used in the current paper express only ERα and not ERβ, ERα seems to be involved. ERs are classically identified as ligand dependent transcription factors. The 5'regulatory regions of E-selectin and ICAM-1 do not contain classical estrogen response element (ERE) sites. Therefore it seems likely that gene transcription is affected by "cross-talk" of ERα with other transcription factors, such as Nf-kB, which is required for TNFα-mediated gene activation and which acts upon a specific site in the 5'regulatory regions of both E-selectin and ICAM-1. Previous studies have reported that E_2 could inhibit nuclear translocation and DNA binding of NF-κB [23] and that ERα could reduce the expression of an NF-κB-driven reporter plasmid [28]. In addition, a human E-selectin promoter study has revealed that the NF-κB site is required for the repressive effect of E_2 [29]. Thus, ERα is likely involved via a non-classical transcription pathway in the E_2 mediated inhibition of TNFα-induced E-selectin and ICAM-1 expression.

To address the question whether the level of ERα is limiting the effect of E_2 in modulating the TNFα response, we decreased endogenously expressed ERα RNA levels in endothelial cells by lentiviral-mediated expression of shERα. Due to integration of the transgene into the genome, silencing was maintained during at least 18 weeks of continuous culturing (data not shown). The 50% knockdown of ERα RNA levels, which coincided with ~50% repression of E_2 induced reporter gene expression did not change the E_2 mediated response towards the expression of adhesion factors. Since ICI blocks the E_2 effect, it is not likely that the E_2 mediated reduction is obtained through an ERα-independent pathway. Probably, the remaining 50% ERα activity is sufficient to inhibit expression of adhesion molecules. Thus, apparently fluctuating ERα levels in endothelial cells do not modulate the responsiveness with regard to E_2 mediated regulation of adhesion molecules. It will be of interest to address the role of ERβ in this process.

In summary, our findings suggest that E_2 has anti-inflammatory properties, as it could down-regulate E-selectin and ICAM-1 expression in endothelial cells. We found that ERα is required, but absolute ERα levels do not determine this anti-inflammatory effect.

Methods

Cell Culture

H5V (a murine endothelial cell line derived from heart) and EOMA (murine hemangioma-derived micro vascular cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 100 units/ml Penicillin, 100 μg/ml Streptomycin and glutamax (Invitrogen) (Complete DMEM). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. In experimental setting to reduce basal E_2 effects, H5V cells were switched to DMEM without phenol red (Gibco BRL) supplemented with 10% charcoal treated FCS (charcoal (Merck) 5gr/ 50ml FCS, mix overnight 4 degrees, centrifuge and filter-sterilize), 100 units/ml Penicillin, 100 μg/ml Streptomycin and glutamax (Invitrogen).

Adhesion experiment

24 hours before treatment, $1{,}5.10^5$ cells were seeded in triplicate in 12-wells plate. Either dissolvant, 10^{-8}M E_2 or 10^{-8}M E_2 + 10^{-6}M ICI was added for an additional 24 hours. 5 hours prior to RNA isolation, the indicated amount of TNFα (GF027, Chemicon) was added (0-1.000 Units). RNA was extracted by 250 μl/well Trizol.

Production of recombinant lentiviruses

The vectors used in our study are so-called SIN vectors, which lose the activity of the promoter located in the 5VLTR upon replication and integration into the genome of the host cells. The Rev-responsive element sequence is recognized by the viral Rev protein and is essential to regulate the production of viral mRNA [37]. The central polypurine tract (cPPT), which is located in the pol region of HIV-1, is retained in the vector as it has been reported to increase nuclear transport of the virus preintegration complex and hence increase transduction efficiency [38,39]. The PRE (posttranscriptional regulatory element) from the human hepatitis B virus (HBV) is a cis-acting sequence that increases expression of transgenes probably by stimulating nuclear export of the mRNA [40]. Expression of the transgene is under the control of an internal promoter: this will be the only mRNA transcribed. The vector plasmids were all derivatives of the pRRL-cPPT-X-PRE-SIN [40]. Plasmids pRRL-cPPT-CMV-GFP-PRE-SIN (here named pLenti-Empty), pRRL-H1 promoter-shERα_1395-cPPT-CMV-GFP-PRE-SIN

(here named pLenti-shERα) and pRRL-H1 promoter-shERα_1103-cPPT-CMV-GFP-PRE-SIN (here named pLenti-shERα_1103) (Figure 3) were constructed with a cytomegalovirus promoter driving the green fluorescent protein. The vectors were produced as described previously [41]. Briefly, the lentiviral backbone containing the gene of interest and the three ‘‘helper’’ plasmids (encoding HIV-1 gag – pol, HIV-1 rev, and VSV-G envelope) were cotransfected overnight using the calcium phosphate method into 293T cells. The medium was refreshed and viruses were harvested after 48 and 72 h, passed through 0.45-Am filters, and stored at -80ºC. Virus was quantitated by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corp., New York, NY, USA) as described [42].

Lentivirus transduction

24 hours before infection, cells were seeded into 96 wells plates (Greiner). H5V at $1{,}5.10^3$ cells/well and EOMA 4.10^3 cells/well. Viral supernatants were added to fresh medium supplemented with 8 µg/ml Polybrene (Sigma), and the cells were incubated overnight. The next day, the medium was replaced with fresh medium. Transduction efficiency was analyzed 3 to 6 days post transduction by FACs analysis. HIV-1 reverse transcriptase inhibitor AZT (GlaxoWellcome) was added to transduced cells at a final concentration of 20 µg/ml.

FACS analysis

For FACS analyses, H5V and EOMA cells were trypsinized gently, the volume was increased by adding PBS/1% FCS, and the cells were kept on ice. The samples were analyzed with a FACScan flow cytometer (Becton – Dick-inson). GFP fluorescence was detected using a 530/30 nm bandpass filter (FL1 channel) following excitation with an argon ion laser source at 488 nm. Using a forward-scatter/side-scatter representation of events, a region was defined to exclude cellular debris from the analysis. A number of events/FL1 (which reflects the fluorescence intensity) histogram was then established according to this region, and percentages of GFP-positive cells were determined in comparison to the negative control (untreated cells). Data analysis was performed using CellQuest 3.1 software (Becton – Dick-inson). For each sample, 10.000 events were collected.

Select GFP positive cells to obtain 100% GFP expressing cell population

EOMA and H5V cells were exposed to shERα expressing lentiviral vectors at MOI 20 and tracking of GFP positive cells monitored the efficiency of gene transfer. Three days post-

infection, a maximum of 50% GFP positive cells was detected. As higher MOI appeared to be toxic, GFP positive cells of the Lenti-Empty, Lenti-shERα transduced population were diluted over 96-wells plate. FACS analysis was performed to select for and pool the cell populations containing near 100% GFP positive cells that consist of the same transgen.

Table 1. Primer sequences of genes used for mRNA quantification

Gene	*Forward primer*	*Reverse primer*
mERα	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
mERβ	5'- TCCTGATGCTTCTTTCTCATGTCA	5'-CACTTCATGCTGAGCAGATGTTC
E-selectin	5'- CCCTGCCCACGGTATCAG	5'-CCCTTCCACACAGTCAAACGT
ICAM-1	5'- GGACCACGGAGCCAATTTC	5'-CTCGGAGACATTAGAGAACAATGC
VCAM-1	5'- ACAAAACGATCGCTCAAATCG	5'-CGCGTTTAGTGGGCTGTCTATC

Real time quantitative PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 μg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [43]. PCR primer sets (TABLE I) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. HPRT was used as the standard housekeeping gene. The significance of differences in relative gene expression numbers C_t ($C_{t(HPRT)}$–$C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a Mann-Whitney U test. Probability values less than 0.05 were considered significant.

Luciferase reporter assay

Transient transfections were performed in triplicate in 12-wells plates ($1,5.10^5$ cells per well) using Lipofectamine (Invitrogen). The effect of lenti-shERα on ERα mediated transcription regulation was determined by co-transfecting the cells with 150ng of reporter construct $(ERE)_3$TATA-LUC and 300 ng pCMV-LacZ. After 24 hours, the cells were stimulated with complete DMEM containing 10^{-8}M E_2 or 10^{-8}M E_2 + 10^{-6}M ICI for an additional 24 hours. The cells were lysed with reporter lyses buffer (Promega) and after centrifugation of 2 min, supernatant was used for determining β-galactosidase normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of cell extract in a monolight luminometer (BD Biosciences). β-galactosidase was measured in a 96-well microtiter plate using the β-Galactosidase Enzyme Assay System in reporter lyses buffer (Promega). Absorbance at 450 nm was determined in a microplate reader. Luciferase activities were normalized for transfection efficiency with the β-galactosidase activity and expressed as a percentage relative to expression levels induced by endogenous estrogen receptor (ER). Expression of endogenous ERα was verified by real time PCR.

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8.

Inhibition of Neointima Formation by Local Delivery of Estrogen Receptor Alpha and Beta Specific Agonists

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ABSTRACT

Objective: Neointima formation is the underlying mechanism of (in-stent) restenosis. 17β-estradiol (E_2) is known to inhibit injury-induced neointima formation and post-angioplasty restenosis. Estrogen receptor alpha (ERα) has been demonstrated to mediate E_2 anti-restenotic properties. However, the role of estrogen receptor beta (ERβ) is not fully elucidated. In the present study, the specific role of vascular ERα and ERβ in neointima formation was assessed.

Methods and results: Neointima formation was induced by placement of a perivascular cuff around the femoral artery of male C57BL/6 mice. E_2 drug-eluting cuff significantly inhibited cuff-induced neointima formation. To address the specific roles of vascular ERα and ERβ on neointima formation, the ERα- and ERβ-selective agonists 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT) and 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) were applied via a drug-eluting cuff. The ERα-specific agonist, PPT, inhibited neointima formation at low but not at high concentrations. Conversely, the ERβ-specific ligand DPN inhibited cuff-induced neointima formation dose-dependently.

Conclusions: Our data demonstrate that, in addition to ERα, specific ERβ activation inhibits neointima formation in a mouse model of restenosis. These data reveal a yet unidentified protective role of ERβ in injury-induced neointima formation.

INTRODUCTION

17β-estradiol (E_2) has been shown to have anti-restenotic properties [1-4]. Nevertheless, the anti-restenotic mechanism of action of E_2 is not fully understood and controversial results regarding its effects on vascular remodelling have been reported [5-8]. This phenomenon may be attributed to the presence of two distinct estrogen receptors (ERs) in the vasculature, ERα and ERβ. ERs are ligand-activated transcription factors [9] and although ERα and ERβ are highly homologous, activation of either one of them may lead to distinct and even opposite biological activities [10-13]. So far, studies in ER knockout mice models have revealed a putative involvement of ERα in the protective effect of E_2 on restenosis [14-17]. Nonetheless, the role of ERβ in mediating the anti-restenotic properties of E_2 has not been fully elucidated.

Recently, ERα- and ERβ-specific agonists have been developed allowing the evaluation of the specific function of each receptor. The novel ERα-specific ligand 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT) is 410-fold more potent in binding to

ERα than to ERβ [18], whereas the 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) binds to ERβ with an 72-fold higher affinity compared to ERα [19]. Therefore, these compounds provide an attractive pharmacological approach to elucidate the biological role of ERβ on neointima formation.

A well-defined mouse model of neointima formation consists of placement of a non-constrictive perivascular cuff around the mouse femoral artery [20,21]. Previously, we showed that the non-constrictive perivascular cuff to induce neointima formation can be constructed from a polymeric formulation suitable for controlled drug delivery. This novel drug-eluting cuff simultaneously induces reproducible neointima formation and allows locally confined delivery of drugs to the cuffed vessel segment [22-25].

In the present study, we assessed the respective role of vascular ERα and ERβ in the anti-restenotic properties of E_2 in a mouse model of restenosis. By local delivery of PPT, an ERα-selective agonist, and DPN, a selective ERβ agonist, we demonstrated that in addition to ERα, ERβ activation leads to neointima formation inhibition in a murine model. These data reveal a yet unidentified protective role of ERβ in injury-induced neointima formation.

RESULTS

3.1. Local delivery of E_2 using E_2-eluting cuffs

3.1.1. E_2 in vitro release profiles

In vitro release profiles of drug-eluting cuffs loaded with 1% and 5% (w/w) E_2 was determined for a three weeks period. E_2 was released in a dose-dependent manner over the 21-day period for both concentrations used (1%: 30.9±14 μg; 5%: 211±14 μg).

3.1.2. Effect of perivascular delivery of E_2 on neointima formation

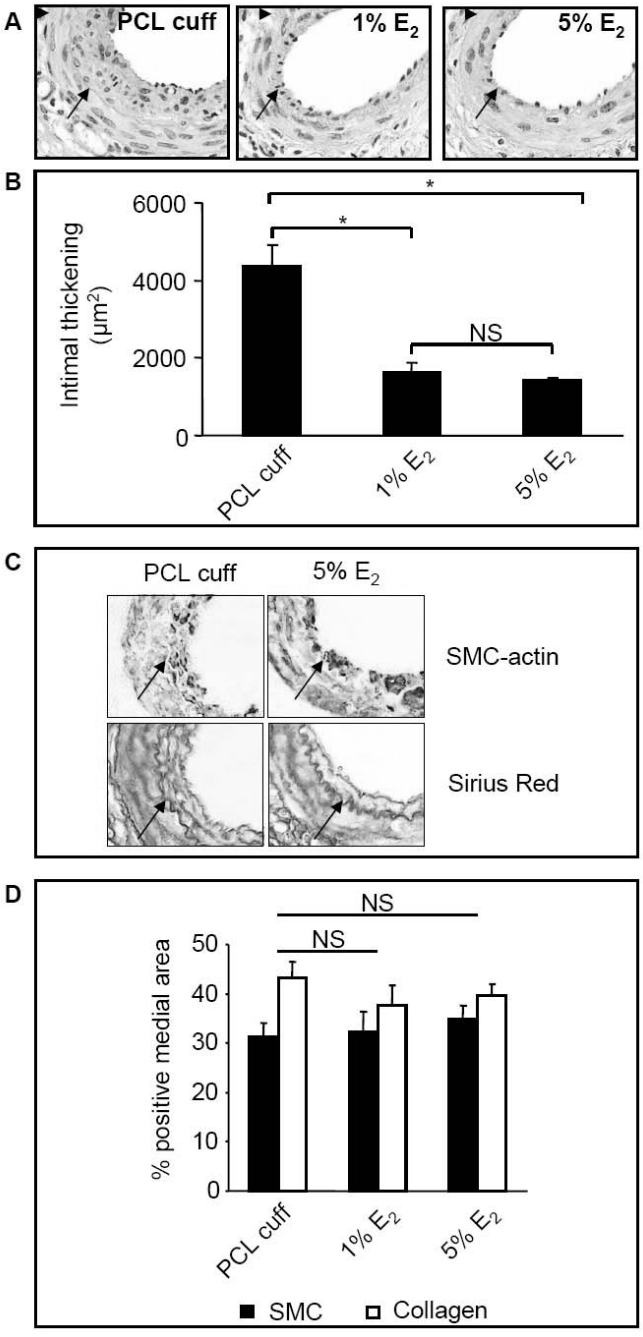

Figure 1.
A: Representative cross-sections of cuffed murine femoral arteries treated with increasing concentrations of E_2 21 days after cuff placement. HPS staining, magnification 400x (arrow indicates the internal elastic lamina; arrowhead indicates the external elastic lamina). B: Total intimal area of cuffed murine femoral arteries 21 days after E_2-eluting cuff placement. Total intimal area was quantified by image analysis using ten sections in each cuffed artery and expressed in μm^2 (mean±SEM, n=6). NS, $P>0.05$ (NS, not significant); *$P<0.05$. C: Representative micrographs of cuffed femoral arteries 21 days after placement of either a control empty cuff (Control cuff) or a 5% (w/w) E_2-eluting cuff (5% E_2). Alpha-SMC actin staining for SMC; similar α-SMC content is observed in both control- and E_2-treated cuffed segments. Sirius red stain for collagen; comparable collagen-positive area is present in both treated and untreated cuffed vessel segments. Magnification 400x (arrow indicates internal elastic lamina). D: Percentage of medial SMC- (close bars) and collagen-positive area (open bars) of cuffed femoral arteries treated with increasing concentrations of E_2 at 21 days after drug-eluting cuff placement. Medial SMC- and collagen-positive area was quantified by image analysis using six sections in each cuffed artery and expressed in μm^2. Mean ± SEM, n=6. NS, $P>0.05$ (NS, not significant).

To assess the effect of local perivascular E_2 delivery on cuff-induced neointima formation, drug-eluting cuffs were loaded with 1% and 5% E_2 and placed around the femoral artery of male C57BL/6 mice for a 21-day period. Microscopic analysis of the cuffed femoral artery

segments revealed that, after three weeks, a concentric neointima had been formed in mice receiving a control drug-eluting cuff. Animals receiving a 1% and 5% E_2-eluting cuff showed a strongly reduced cuff-induced neointima formation development (Fig. 1A). Morphometric analysis revealed a significant inhibition of cuff-induced neointima formation between mice receiving a control drug-eluting cuff and animals receiving an E_2-eluting cuff (Fig. 1B). Likewise, E_2 perivascular treatment resulted in a significant decrease in intima/media ratios for both E_2 loading dosages (Control: 0.43±0.07; 1%: 0.17±0.04, P=0.005; 5%: 0.18±0.02, P=0.003) as compared to control drug-eluting cuff. In addition, no toxic effects of local perivascular delivery of increasing E_2 concentrations on vascular integrity were found as determined by quantification of medial SMC and collagen content (Fig. 1C and 1D).

3.2. ERα and ERβ expression in cuffed femoral arteries

E_2 may exert its inhibiting effects on neointima formation via both vascular ERα and ERβ. As depicted in Fig. 2A, both ERs mRNA levels were upregulated time-dependently after the induction of the stenotic process. ERs mRNA levels showed a peak expression 7 days after cuff placement (59.5±3.9-fold increase for ERα vs. 11.4±4.2-fold increase for ERβ, both P<0.05) compared with control sham-operated arteries (T=0 days), after which the signal declined. In addition, immunohistochemical analyses showed that, both ER subtypes are present in murine femoral arteries (ERα: 19.2±0.5%; ERβ: 48.4±6.8%, Fig. 2B and 2C). Moreover, during the cuff-induced neointima formation process, cuffed femoral arteries expressed both ERα and ERβ also in intimal tissue (Fig. 2B and 2C). Altogether, ERβ is more abundantly present in vascular tissue as shown by immunohistochemistry analysis. On other hand, upregulation of ERα expression is more prominent upon vascular injury. Thus, both ERα and ERβ are present and have the potential to contribute to the anti-restenotic properties of E_2.

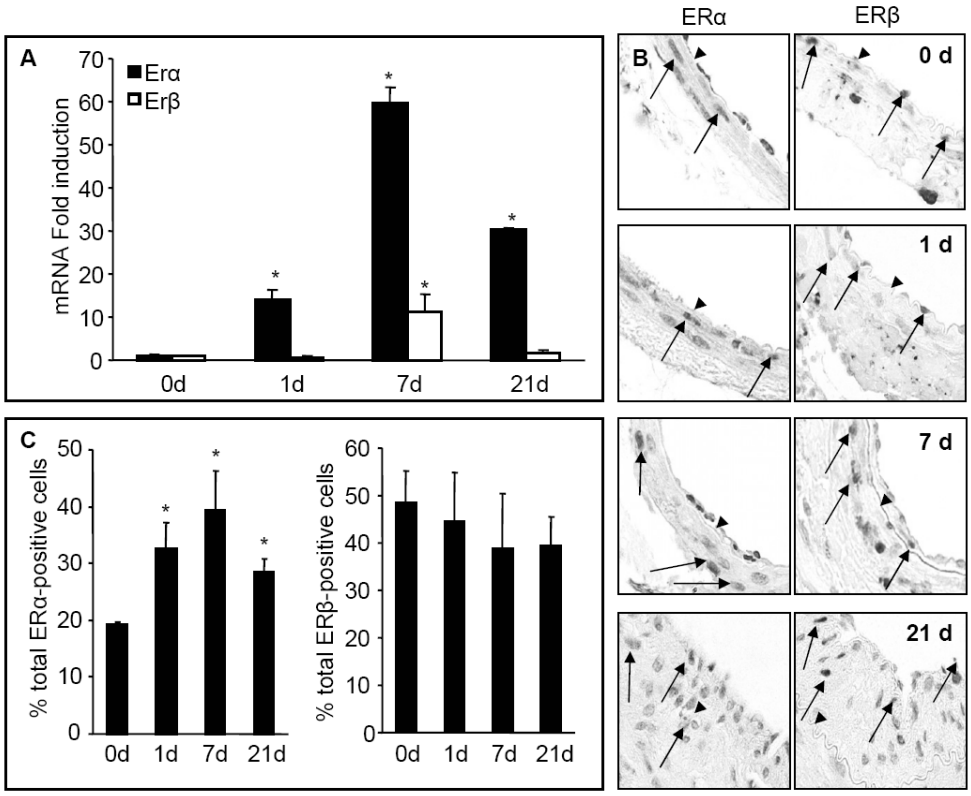

Figure 2. A: Fold induction of ERα and ERβ mRNA in cuff-induced neointima formation in time (mean±SEM, n=4; **P*<0.05 as compared to T=0 days). B: ERα and ERβ localization in cuffed murine femoral artery on diverse timepoints. Both ERα and ERβ were present on medial tissue and endothelial cell monolayer in intact arteries (0 days). During the process of neointima formation development (1, 7, and 21 days) ERs expression was also present in intimal tissue. Magnification 400x. Arrowhead indicates internal elastic lamina; arrow indicates ERs positive cells. C: Percentage of total ERα- and ERβ-positive cells of cuffed femoral arteries after cuff placement. ERα- and ERβ-labeled cells were counted in six equally spaced cross-sections from each cuffed artery and expressed as a percentage of the total number of cells (mean±SEM, n=6; **P*<0.05 as compared to T=0 days).

3.3. Local specific activation of ERα and ERβ in femoral arteries

3.3.1. PPT and DPN in vitro release profiles

To examine whether PPT and DPN were suitably loaded and released from our drug delivery device, the in vitro release profiles of 0.5%, 1%, 2.5% and 5% PPT- and 1% and 5% DPN-eluting cuffs were assessed. PPT showed a sustained and dose-dependent release for the 21-day period (0.5%: 16±0.4 μg; 1%: 36±2 μg; 2.5%: 68±1 μg; 5%: 160±6 μg). DPN was

also released from the drug-eluting cuffs in a dose-dependent manner over time. In total, 33±1 μg was released from the 1% and 83±3 μg from the 5% DPN-eluting cuffs, respectively.

3.3.2. Effect of PPT- and DPN-selective ER subtypes activation on neointima formation

To assess the role of ERα in the E_2-mediated inhibition of cuff-induced neointima formation, drug-eluting cuffs were loaded with 0.5%, 1%, 2.5%, and 5% PPT, a highly specific ERα ligand, and placed around the femoral artery of mice for three weeks. It should be noted that a broader concentration range of PPT was used as compared to E_2 and DPN. This was due to the seemingly contrasting data observed with the 1% and 5% PPT-eluting cuffs on neointima formation, as discussed below.

In animals receiving a control drug-eluting cuff a neointima had been formed. Remarkably, morphometric quantification revealed only a significant inhibition of cuff-induced neointima formation in the cuffed segments treated with the lowest PPT concentrations. Cuffed arteries locally treated with higher PPT concentrations (2.5 and 5%) did not show an inhibitory effect on neointima formation as compared with control cuffed arteries (Fig. 3A and 3C). Likewise, only intima/media ratios of the PPT-treated arteries with the lowest concentrations were significantly decreased (Control: 0.42±0.07; 0.5%: 0.13±0.01, $P<0.001$; 1%: 0.20±0.03, $P=0.008$; 2.5%: 0.34±0.05, $P=0.5$; 5%: 0.56±0.05, $P=0.2$) as compared to controls.

By placing a 1% and 5% (w/w) DPN-eluting cuff around the femoral artery of male C57BL/6 mice for 21 days, the role of ERβ on neointima formation was assessed. Morphometric analysis of the cuffed arteries of both DPN-treated groups showed a significant inhibition of neointima formation as compared to control cuffed segments (Fig. 3B and 3D). Moreover, intima/media ratios of the DPN-treated groups were also significantly decreased (Control: 0.42±0.07; 1%: 0.22±0.05, $P=0.02$; 5%: 0.15±0.03, $P=0.001$) as compared to controls.

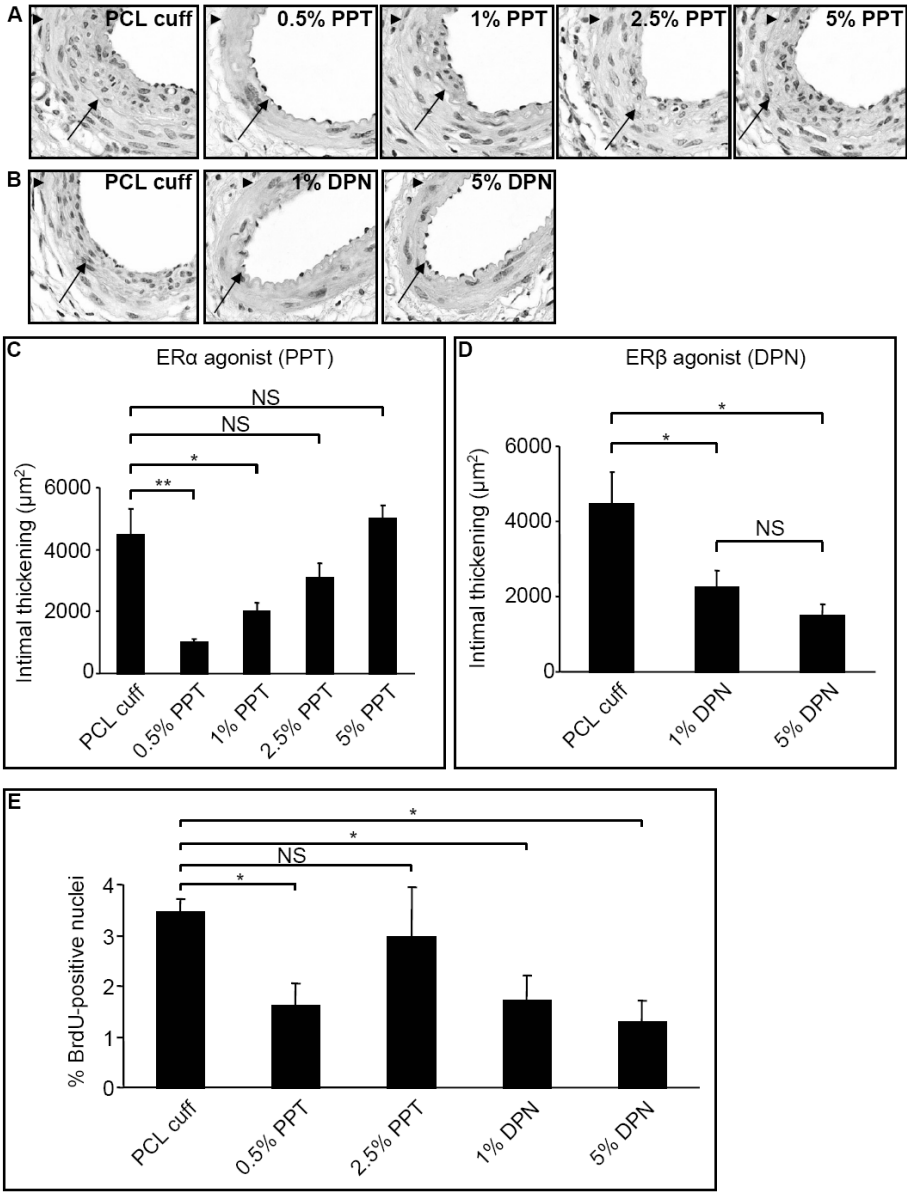

Figure 3. Representative cross-sections of cuffed murine femoral arteries treated with increasing (A) PPT and (B) DPN concentrations 21 days after cuff placement. HPS staining, magnification 400x (arrow indicates the internal elastic lamina; arrowhead indicates the external elastic lamina). Total intimal area of cuffed femoral arteries 21 days after (C) PPT- or (D) DPN-eluting cuff placement. Total intimal area was quantified by image analysis using ten sections in each cuffed artery and expressed in μm^2 (mean±SEM, n=6). NS, $P>0.05$ (NS, not significant); *$P<0.05$; **$P<0.01$. E: Percentage of BrdU-positive cells in cuffed femoral arteries treated with increasing concentrations of PPT (0.5 and 2.5%) and DPN (1 and 5%) 21 days after drug-eluting cuff placement. BrdU-labeled nuclei were counted in six equally spaced cross-sections from each cuffed artery and expressed as a percentage of the total number of nuclei. Mean±SEM, n=6. NS, $P>0.05$ (NS, not significant); *$P<0.05$.

To further investigate the apparent discrepancy on cuff-induced neointima formation between animals perivascularly treated with either an ERα or an ERβ specific agonist, DNA synthesis was evaluated. Cellular proliferation was assessed by examining incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA at 21 days after cuff placement in mice receiving either a control drug-eluting cuff, a PPT- (0.5% and 5%) or a DPN-eluting cuff (1% and 5%). As depicted in Fig. 3E, a profound incorporation of BrdU was observed 21 days after surgery in cuffed vessel segments receiving a control drug-eluting cuff (3.45±0.25%). In line with the morphometric analysis, only the 0.5% PPT-eluting cuff showed a decreased cellular proliferation but not the higher PPT dosage (0.5%: 1.62±0.43%, *P*=0.02; 2.5%: 2.95±1.01%, *P*=0.18). Conversely, cuffed artery segments of mice receiving either a 1% or a 5% DPN-eluting cuff showed a significantly decreased cellular proliferation as compared to control cuffed arteries (1%: 1.71±0.50%, *P*=0.016; 5%: 1.27±0.43%, *P*=0.016).

DISCUSSION

The present study evaluates the respective roles of vascular ERα and ERβ on neointima formation. Here we show, for the first time, the effects of specific ER subtype ligands on cuff-induced neointima formation in the mouse femoral artery. Local E_2 treatment resulted in a substantial and significant inhibition of cuff-induced neointima formation (Fig. 1). Surprisingly, mice receiving the PPT-eluting cuffs displayed a significant reduction on neointima formation only for the lower PPT concentrations (0.5%: 78±3%; 1%: 56±8%) but not for the 2.5% and 5% PPT-eluting cuffs. Conversely, perivascular delivery of DPN displayed an inhibitory effect on cuff-induced neointima formation at both low and high concentrations (1%: 50±10%; 5%: 67±7%) (Fig. 3).

E_2 has been shown to have vasoprotective properties. In rats, systemic E_2 therapy resulted in reduced vascular SMC proliferation and migration, which are fundamental steps in restenosis development [5]. In porcine coronary arteries, it has been shown that local delivery of E_2 decreases post-angioplasty restenosis due to endothelial function improvement [26-28]. Furthermore, the first short-term human pilot study using E_2-eluting stents showed low rates of restenosis [29].

E_2 mediates its effects primarily via interaction with its receptors ERα and/or ERβ. Recently, we have generated a drug-eluting polymer cuff which enables local delivery of compounds to the vasculature in an established mouse model of restenosis [22-25]. In the present study we demonstrate that both ER subtypes are expressed in cuffed femoral arteries

during the process of neointima formation (Fig 2). Therefore, both ER subtypes may be accountable for the inhibitory effect of E_2 on cuff-induced neointima formation.

Thus far, the specific role of vascular ER subtypes in the vascular wall is not fully elucidated. The current knowledge of the respective role of vascular ER subtypes derives almost exclusively from ERα- and ERβ-null mouse models [14-17]. Although ER-null mice provide interesting clues, they imply several shortcomings. Due to whole body ER deficiency, direct vascular effects of E_2 cannot be discriminated from systemic effects. In addition, potential compensatory mechanisms may have occurred during development. In the past, Pare and colleagues [17] have demonstrated very elegantly that in ERα-null mice E_2 does not have a protective effect on injury-induced vascular remodelling. However, although the concentration of E_2 used in these studies are optimal for activating ERα (mean circulating levels of 0.33 to 0.43 nmol/L) it might be suboptimal on activating the ERβ (Kd for ERα=0.2 nM and for ERβ=0.5 nM).

To dissect the contribution of ERα and ERβ in preventing neointima formation the ERα- and ERβ-selective agonists, PPT and DPN, were used. The selectivity of PPT and DPN for both receptor subtypes enables detailed analysis of the contribution of both ERs to the protective effects on neointima formation in the current experiments. PPT induces exclusively ERα mediated transcription and not ERβ (Kd for ERα=0.4 nM and for ERβ=417 nM) [18]. Thus, PPT can be stated as a highly selective ERα agonist. In vivo, we demonstrated that local release of PPT led to either anti-restenotic effects or no effect on restenosis, as low dosages inhibited neointima formation whereas high concentrations did not.

On the other hand, DPN displays ERβ specificity (Kd for ERα=80 nM and for ERβ=2.8 nM) [19]. Also in vivo DPN seems to act like a specific ERβ agonist. For example, systemic administration of the relatively high dose of 1 mg/kg/day DPN to rats does not alter uterine weight, which is regarded as a true ERα target tissue [30]. In the present study, both low and high concentrations of DPN led to an inhibition of neointima formation. Therefore we can state that, in this model, activation of ERβ seems to have a protective effect on injury-induced neointima formation.

The surprising finding that PPT does not seem to have a protective effect on cuff-induced neointima formation at higher dosages suggests a so called bell-shaped response curve, often seen when nuclear receptors are activated, might occur also in case of PPT-mediated activation of ERα. However, since E_2 activation does not show this response curve in our present studies and the Kd for the ERα for E_2 and PPT are similar (0.2 and 0.4 nM,

respectively) we do not believe this is the explanation for the observed phenomenon. Currently, the reason for the observed response curve for PPT is not fully understood.

In conclusion, while literature proposes ERα as the major receptor involved in the anti-restenotic and anti-atherosclerotic effects of E_2. Our data provide evidence for a yet unidentified protective role of ERβ in injury-induced neointima formation as well. Nevertheless, there seem to be complex and dose-dependent opposite roles for ERα and ERβ in vascular tissue.

METHODS

2.1. Drug-eluting cuffs and in vitro release profiles

E_2 was purchased from Sigma Diagnostics (St Louis, USA). PPT and DPN were obtained from Tocris Cookson Ltd. (Bristol, UK). Poly(ε-caprolactone)-based drug delivery cuffs were manufactured as previously described [22]. Drug-loaded cuffs were made from the different blended molten drug-polymer mixtures and designed to fit around the femoral artery of mice. Drug-eluting cuffs had the shape of a longitudinal cut cylinder with an internal diameter of 0.5 mm, an external diameter of 1.0 mm, a length of 2.0 mm and a weight of approximately 5.0 mg.

Drug-eluting cuffs were loaded with 1% and 5% (w/w) E_2, with 1% and 5% (w/w) DPN and with 0.5%, 1%, 2.5%, and 5% (w/w) PPT. In vitro release profiles (n=5/group) were performed by UV-VIS absorbance methods (225nm, 235nm and 257nm, respectively) as described elsewhere [22]. Calibration graphs of the different compounds were established by measuring the absorbance of a set of standards of each compound in the 0-50 μg/ml concentration range.

2.2. Femoral artery cuff mouse model

For experiments, 10-12 weeks old male C57BL/6 mice were used. Animals were fed a standard chow diet (R/M-H, Ssniff, Soest, Germany). At the time of surgery, mice were anaesthetized with an intraperitoneal injection of 5 mg/kg Dormicum (Roche, Basel, Switzerland), 0.5 mg/kg Dormitor (Orion, Helsinki, Finland) and 0.05 mg/kg Fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and loosely sheathed with a non-constrictive cuff [21,22]. Either a control empty cuff, an E_2-eluting cuff (1% and 5% w/w), a PPT-eluting cuff (0.5%, 1%, 2.5%, and 5% w/w), or a DPN-eluting cuff (1% and 5% w/w) was used (n=6/group).

All animal work was approved by TNO institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Quantification and histological assessment of intimal lesions in cuffed femoral arteries

Animals were sacrificed 21 days after cuff placement. Histological analyses were performed as described previously [21-23]. All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert´s elastin staining was used to visualize elastic laminae.

Smooth muscle cells (SMC) were visualized with α-SMC actin staining (1:800, Roche). Collagen content was determined using Sirius red stained sections. The amount of medial SMC and collagen content was determined by morphometry (Leica Qwin, Wetzlar, Germany) and expressed as the percentage of total medial area consisting of SMC actin- or Sirius red-positive area in six equally spaced serial cross-sections in all animals [23].

Incorporation of 5-bromo-2´-deoxyuridine (BrdU) into DNA as a marker of DNA synthesis was used to determine the rate of cell proliferation in cuffed vessel segments. Mice (n=6/group) were injected i.p. with 25 mg/kg BrdU (Sigma Diagnostics) three times at 72, 48, and 24 hours prior to sacrifice. Sections were incubated with a mouse monoclonal anti-BrdU antibody (1:50; DakoCytomation, Glostrup, Denmark). Specimens incubated with a mouse isotype-matched IgG diluted to the same concentration as the primary antibody were use as control. The number of BrdU-labeled nuclei per cuffed artery were counted in six equally spaced cross-sections and expressed as a percentage of the total number of nuclei.

2.4. Estrogen Receptors in femoral arteries

The presence of ERα and ERβ in cuffed vessel segments was visualized by immunohistochemistry using a rabbit and goat primary polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA) against the mouse ERα (1:600) and ERβ (1:100), respectively, according to the manufacturer's instructions.

Immunohistochemical analysis were performed in paraffin-embedded femoral artery segments at 0, 1, 7, and 21 days after cuff placement (n=6/timepoint). Specimens incubated with a mouse isotype-matched IgG diluted to the same concentration as the primary antibody was used as control. ERα- and ERβ-positive cells were counted in six equally spaced cross-sections in all mice and expressed as a percentage of the total number of cells.

2.5. Real time RT-PCR mRNA analysis

Mice underwent femoral artery cuff placement as described. Animals were sacrificed at different timepoints after surgery (0, 1, 7, and 21 days), 4 mice for each timepoint. Femoral arteries were isolated, harvested and snap frozen. Total RNA was isolated using the RNeasy Fibrous Tissue Mini-Kit (Qiagen, Venlo, The Netherlands) according to manufacturer's protocol. Of all RNA samples cDNA was made using Ready-To-Go RT-PCR beads (Amersham Biosciences, Uppsala, Sweden).

Intron-spanning primers and TaqMan® probe were purchased from TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, USA). HPRT (hypoxanthine phosphoribosyltransferase) was used as a housekeeping gene. For each timepoint RT-PCR was performed in duplicate. Per timepoint the signals were averaged and the average signal of the housekeeping gene HPRT was subtracted (ΔCt). ΔΔCt was determined as the difference between ΔCt values of the control sham-operated arteries (0 days) and the cuffed femoral arteries. Data are presented as fold induction (normalized to T=0 days), which was calculated as $2^{-\Delta\Delta Ct}$ [25].

2.6. Statistical analysis

Results are expressed as mean±SEM. Data were analyzed using the Mann-Whitney *U* test (SPSS 11.5). A value of $P<0.05$ was considered statistically significant.

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9. Summary, Discussion & Perspectives

Summary

A protective function of E_2 in vascular health has been established [1-3]. Clinical and experimental studies, however, have demonstrated that estrogens are not a magic bullet, but have both beneficial and detrimental effects on different organ systems. Therefore, more insight into the mechanistic actions of estrogens and the respective role of ERα and ERβ in individual cell types is required. To address some of these issues, we set out to modulate ERα and ERβ signaling tissue-specifically.

Hepatic E_2 signaling

Elevated plasma lipid levels are strongly associated with an increased likelihood to develop atherosclerosis. Studies in both humans and animal models indicate that long–term E_2 treatment reduces hyperlipidemia. Post-menopausal hormone replacement therapy (HRT) changes the lipid profile in a potentially anti-atherogenic direction by reducing LDL-cholesterol and triglyceride levels and increasing HDL-cholesterol levels [4]. Mouse models in which E_2 signaling is disrupted, e.g. ovariectomy, ArKO and ERα$^{-/-}$ mice, develop hypercholesterolemia upon aging [5-7].

The liver plays a pivotal role in lipid homeostasis. We hypothesized that the effects of E_2 on lipid metabolism are predominantly regulated by hepatic ERα. ER mediated signaling is known to be complex and controlled via tightly regulated pathways. Therefore, to assess the specific role of hepatic ERα mediated action, subtle modulation is required. In **chapter 2** we approached this by making use of RNA interference (RNAi). Thus far a relatively limited number of studies have described the successful application of RNAi technology in vivo. We obtained efficient intracellular delivery of short hairpin (sh)RNA's that were targeted against mouse ERα (shERα) specifically in liver of adult mice by utilizing Ad vectors. E_2 mediated hepatic ERα activity was reduced 60% by Ad.shERα for at least five days. This was demonstrated by an in vivo reporter mouse model that expresses luciferase driven by a promotor that contains estrogen response elements. Binding of the liganded ERα thus results in luciferase expression that can be detected in vivo using an ultra-sensitive CCD camera. Thus, Ad vectors were demonstrated to be an effective strategy to deliver shRNA molecules to the liver of mice to reduce gene expression. Subsequently, Ad.shERα vectors were applied to further investigate the function of hepatic ERα.

The role of hepatic ERα in lipid metabolism was explored in APOE3-Leiden female mice by applying Ad.shERα (**chapter 3**). While hepatic ERα RNA and protein levels were

reduced by 60%, hepatic and serum TG and Chol levels, as well as the VLDL-TG production rate were not affected. In contrast, expression of some lipid related genes, like Cyp7a, PPARα and SHP gene were changed. Apparently, the changes in the expression of these lipid related genes is compensated for by alternative transcriptional or post-transcriptional mechanisms and does not affect plasma lipid levels. Thus, since reduced hepatic ERα levels did not result in a clear lipid phenotype, our data imply that hepatic ERα is not a limiting factor in hepatic lipid metabolism.

E_2 has also been implicated in the regulation of glucose homeostasis and insulin sensitivity. Interestingly, the risk factors hyperlipidemia and insulin resistance both increase post-menopausally. HRT has been associated with a reduction in the incidence of diabetes [8,9]. Insulin sensitivity was improved by several weeks of E_2 treatment in mice deficient for E_2 [10]. However, the beneficial effects on insulin sensitivity obtained by long-term E_2 treatment could very well be an indirect consequence of the E_2 induced changes in for example adipose tissue. To gain insight in the direct and liver-specific effects of E_2, we determined the acute effects of E_2 on insulin sensitivity. In chapter 4, we performed a hyperinsulinemic/euglycemic clamp study in an insulin resistant mice model, six hours after E_2 treatment. In this short time span, hepatic glucose production was improved, while as expected total bodyweight and hepatic lipid content, known contributors of hepatic insulin sensitivity, were unchanged. Apparently, E_2 influences glucose homeostasis directly, resulting in an improvement of hepatic insulin sensitivity. Since ERα mediated transcription is mainly induced in liver and transcription levels of genes involved in hepatic glucose production were repressed six hours after E_2 treatment, we assume that hepatic E_2 signaling is responsible for these effects. Thus, our data imply a relatively major role for E_2 in regulating hepatic glucose production.

E_2 signaling in the vasculature

Beyond the effects on metabolic parameters that could account for the beneficial effect of E_2 on vascular diseases, several observations suggest a direct effect of E_2 on the vessel wall. To obtain a better insight in the direct effects of E_2 on the vessel wall and the relative importance of ERα and ERβ, we set out to achieve local and time-controlled modulation of ER signaling within the vascular tissue.

Vascular gene transfer

Studying of the ER subtype specific signaling in vascular cells is challenging. Firstly, in culture most vascular cells lose their ERs. Secondly, gene transfer to introduce exogenous ERs is cumbersome. Vascular cells are notoriously resistant to both non-viral as well as viral gene transfer. In **chapter 5**, the transduction efficiency of vascular cells in vitro has been improved by targeting Ad vectors using a linker protein. This linker construct consisted of the extra cellular domain of the coxsackie virus Ad receptor (CAR) genetically fused to avidin. Via its avidin moiety, a biotinylated cyclic RGD peptide was bound. This resulted in a targeting construct that binds to the Ad vector with one side and to $\alpha_v\beta_{3/5}$ integrins with the other side. This targeting strategy is relatively fast and does not require rederivation of the Ad vectors (as is required for genetically retargeted Ad). The redirection of Ad specificity from cellular CAR to integrins resulted in a significantly enhanced gene transfer to both transformed as well as primary vascular cells in vitro (**chapter 5**). Thus, our retargeting strategy renders Ad vectors exquisitely applicable for the analysis of gene function in vascular cells in vitro.

Next, an extensive effort was made to apply the cRGD-equipped Ad vectors *in vivo* (**chapter 6**). Normally, after systemic application, Ad5 (the most commonly used Ad variant) vectors sequesters in the liver, hampering delivery to alternative target tissues. By using the previously described targeting construct we were able to overcome this barrier, as de-targeting of the liver was consistently found. However, gene delivery to normal and injured carotid arteries was never observed after systemic administration of targeted Ad vectors. This is likely due to rapid clearance of Ad from the bloodstream and the anatomical position of the carotid artery. However, local incubation of cRGD-Ad in both normal and injured carotid artery also did not resulted in enhanced gene transfer. Thus, in principle enhancement of vascular gene transfer should be achievable (**chapter 5**). However unknown parameters have thus far prevented in vivo vascular gene transfer.

Role ER in the vascular wall

An important role in the anti-atherogenic effect of E_2 seems to be fulfilled by the endothelium [11]. In **chapter 7**, we demonstrated the repressive effect of E_2 on cytokine-mediated induction of adhesion molecules like E-selectin and ICAM-1 in two independent vascular cell lines. To determine the role of ERα, we repressed ERα in endothelial cell lines by lentiviral mediated expression of shERα. A significant reduction of ERα mRNA levels (60%) with an equivalent reduction in its activity was observed in the sub-cloned cell lines

that contain near 100% transduced cells. Using these cell lines, we demonstrated that the level of ERα does not limit the repressive effect of E_2 on expression of adhesion molecules.

In vivo, both ERα and ERβ are expressed in the vessel wall. It has been demonstrated that although ERα and ERβ share homologous domains, they can exert distinct and sometimes opposite biological action. Therefore, we postulated that the biological effects of E_2 on vascular remodelling are the result of the expression levels and balance between ERα and ERβ levels in vascular tissue. In **chapter 8,** we addressed the role of both endogenously expressed ERs in vivo on neointima formation by drug-releasing non-constrictive polymeric cuffs. Using these devices, a restenosis-like lesion is induced and ER subtype specific agonists are released. Interestingly, local release of the dual agonist, E_2 and the ERβ selective ligand, DPN both significantly reduced neointima formation. On the other hand, inhibition of intimal hyperplasia when solely ERα was activated was only observed after release of low concentrations. Our results demonstrate distinct, and partly opposite responses of ERα and ERβ on neointima formation. In contrast to the ERα and ERβ knockout studies, which propose that ERα is the receptor responsible for the anti-restenotic and anti-atherosclerotic effects of E_2 [12-15], our data provide evidence for an important, thus far unnoticed role of vascular ERβ in the prevention of restenosis.

Discussion & Perspectives

ER mediated cellular processes are very complex. To unravel the role of either ERα or ERβ, whole body ER deficient mice have been generated. However, information obtained from whole body ER deficient mice needs to be interpreted with caution. The complex phenotype of knock-out mice could obscure the role of ER at later stages of development or in specific tissues. In addition, back-up mechanisms that counterbalance the ER deficiency might be induced. With the development of conditional knock-out technology, these problems can be addressed [16]. However, this technology requires considerable effort and time. As an alternative method for inhibiting target gene expression, we have generated shERα constructs that are described in this thesis. These shERα constructs can be applied at a specific time point during development. By using a suitable vector, tissue specificity can be achieved. However, for all RNAi based approaches, the percentage and type of cells that can be transduced with a specific vector and the knock-down efficiency are variables that need to be taken into account when interpreting the results. In this respect, adenovirus vectors are highly

suitable for both dividing and non-dividing cells and in general are capable of inducing high levels of transgene expression.

The vessel wall seems to be an important target tissue for E_2. However, low efficiency of available gene-transfer systems, limits the applicability to dissect the role of ERs in vascular cells. Generally, viral vectors are more successful as compared to non-viral vectors in transducing vascular cells. In **Chapter 5**, it has been demonstrated that by re-targeting Ad vectors to integrins, gene transfer to primary vascular cells could be enhanced considerably. However, although, efficient gene transfer was accomplished in primary VSMC and ECs, gene transfer was not enhanced in the vessel wall in vivo. Even after local incubation in an injured artery, enhanced gene transfer was not observed (**chapter 6**). It is possible that the physical size of the targeted Ad vector, in combination with the dynamics of integrin expression in injured vessels is incompatible with the incubation time of Ad (10 minutes). However, it should also be noted that approaches to enhance Ad mediated gene transfer to vascular cells in vivo, thus far has resulted in very few successful applications. Therefore, we believe that essential knowledge regarding the fate and mechanism involved in the uptake of Ad in tissues other than liver in vivo is missing. This hampers the construction of an efficient, specific and non-toxic delivery device. Thus, basal research on Ad vectors should continue.

The understanding of E_2 action is incomplete and much remains to be discovered with respect to the effects of the large changes in E_2 concentrations and ER levels in development and aging. Thus far, the effect of different ERα levels on metabolic parameters and in vascular tissue is unknown. In this thesis, the role of ERα levels has been addressed by use of a shERα construct (**chapter 2**). This shERα construct allowed studying the response of E_2 in specific target tissues in the presence of altered ER levels. It revealed that hepatic ERα levels are not rate-limiting in determining plasma and liver lipid parameters (**chapter 3**) and that vascular ERα levels do not limit the repressive effect of E_2 on adhesion molecule expression (**chapter 7**). In human vascular tissues, it has been reported that the expression of ER changes with pathological conditions such as atherosclerosis [17,18]. Although ER knock-out mouse models have shown that ER deficiency leads to abnormal vascular function [19], it is not known whether reduced ERα levels cause a predisposition towards vascular dysfunction. The results obtained from our in vitro experiments, imply that reduced ERα levels are not a causative factor. However, more research is required to verify this hypothesis. As a follow-up

study, it would be interesting to screen for genes whose expression levels are more susceptible to ERα quantities. These genes can be identified by performing micro-array analysis using the shERα expressing endothelial cell lines. Furthermore, the effect of reduced ERα levels on expression of adhesion molecules and its effect on the development of the atherosclerotic process remain to be addressed in an in vivo model.

Our observation that lipid parameters were not changed upon shERα treatment, is in line with the fact that the changes with respect to lipid metabolism have not been reported in ERα heterozygous knockout mice. In homozygous ERα knockout mice the effects are only apparent under stressed conditions and/or upon aging [5,7]. Our data imply that the absence of a lipid phenotype in young mice is not due to compensatory changes, like for instance up-regulation of ERβ as a result of the ERα deletion, but truly indicate that hepatic ERα levels are not limiting. In addition, changes in lipid parameters induced by systemic E_2 administration or after ovariectomy are only apparent after a time lag of at least two weeks (personal communication, d'Olivera, Hoekstra). Therefore, it seems likely that the changed plasma lipid levels induced by prolonged E_2 administration are initiated by a cascade of events, in which non-hepatic tissues, like brain and adipose tissue play an important role. To address these issues, E_2 signaling should be modulated in a tissue specific manner. At the moment, techniques to apply drugs or RNAi locally are available. In rat models it has been shown that RNAi can be applied into certain regions of the brain [20]. In adipose tissue, E_2 signalling could be modulated by transplantation of fat from either $ER\alpha^{-/-}$ or $ER\beta^{-/-}$ mice.

The observed changes in lipid metabolism induced by long-term modulation of E_2 signaling could also be an indirect consequence of the short-term and perhaps prolonged changes in hepatic insulin sensitivity (**chapter 4**). The dissection of these cause and effect relations would require a substantial effort. As an initial study, the hepatic glucose pathway that is targeted by E_2 should be mapped using both transcriptomic (gene expression levels) as well as proteomic approaches (protein levels and modifications). By blocking parts of pathways that are thus revealed, the effect of prolonged E_2 administration on hepatic glucose production and subsequent changes in lipid parameters could be assessed.

The doses of E_2 that have been applied in reported experimental as well as in clinical studies are highly variable. However, the effect of these different E_2 levels in vascular tissue is unclear. In **chapter 8**, different concentrations of ER subtype selective ligands were

released to locally activate either ERα or ERβ. These data revealed that both a low and high dose of the dual agonist E_2 and the ERβ selective agonist, DPN, inhibited neointima proliferation (**chapter 8**). Thus for these two ligands, a dose-dependent effect was not observed. However, an inverse dose-dependent effect was observed when the ERα specific agonist, PPT was applied. PPT significantly inhibited neointima proliferation at low dose but not at high dose. Inverse dose-dependent effects could be explained by a dose-dependent shift in ERα:ERβ activity. This seems unlikely, since a dose dependent effect was only observed with the ERα specific agonist, PPT and not with the dual agonist E_2. An alternative exlanation for the inverse dose-dependent effect of PPT could be PPT mediated down-regulation of ERα expression. A high dose of PPT would result in very low ERα expression levels and thus a decrease in ERα-mediated effects.

The dose-dependent effects of PPT can also be explained by ER subtype specific biological effects. ERα might play an important role in the balance between pro- and anti-restenotic pathways. At low ERα activity, the anti-restenotic effects could be dominant, but also maximally induced. Increasing ERα activity by applying a high dose of PPT will then only enhance pro-restenotic effects and not the anti-restenotic effects.

It is obvious that there is a complex interplay between pathways induced by either ER subtype. Most likely, the interplay between the ER induced pathways in the vasculature is also affected by devevelopmental stage, aging and pathology such as restenosis and atherosclerosis. In conclusion, this study indicates that the dosing of ER ligands may be critical in determining the magnitude and direction of the biological effects.

The results obtained from **chapter 7 & 8** imply that E_2 prevents atherosclerosis by interfering either prior to injury or very early post-injury. In **chapter 7** we observed that pre-treatment with E_2 significantly reduces the cytokine-induced expression of the endothelial adhesion molecules E-selectin and ICAM-1. Suggesting that due to E_2, the endothelial cells are less responsive to inflammatory signals. Opposite results have also been published [21,22]. However, in those studies, E_2 was added simultaneously with the cytokine instead of before treatment. In **chapter 8**, E_2 and DPN both significantly inhibited neointima proliferation. When the drug release profiles are taken into account, both E_2 and DPN are only released in the first week. In addition, the half-life of both compounds is less than a day [23,24]. Thus, from these studies it seems likely that the anti-inflammatory and anti-restenotic effects are exerted pre-injury. Our data do confirm earlier studies in primates, rats and rabbits,

which have demonstrated that the protective effects of E_2 are only apparent if E_2 was administered prior to the development of atherosclerosis and not when arterial damage was present prior to hormone treatment [25-29]. For future study, it would be interesting to address this point in more detail. Because of the applicability of local drug treatment, our mouse model would offer the opportunity to perform such a time range. In addition to releasing ER subtype specific ligands simultaneously with the induction of restenosis (**chapter 8**), restenosis can first be induced by applying an empty cuff, which is then followed by a drug-releasing cuff. To address the local and time dependent effect of E_2 on atherosclerosis, these cuffs should be used in an atherosclerotic mice model, such as $ApoE^{-/-}$ and ApoE3Leiden mice.

Clinical perspectives

Although caution should be taken when extrapolating results obtained by *in vitro, ex vivo* or animal models to humans, findings obtained by numerous experimental studies clearly indicate the importance of E_2 status on vascular endothelial function**.**

Atherosclerosis is multi-factorial by nature, caused by a wide variety of genetic as well as environmental factors, and develops decades earlier than its clinical manifestation. Therefore, drug therapy alone to treat the atherosclerotic vessel wall is not likely to be sufficient. Management of risk factors, like obesity, hyperlipidimea, hypertension and insulin resistance should be the primary approach to decrease the development of atherosclerosis. This can be achieved by lifestyle modifications (ie, weight control, change in diet, regular exercise and smoking cessation). Whenever this is not feasible, because of for example a genetic predisposition to hyperlipidemia, drug therapy becomes the primary approach.

E_2 has been suggested as a possible drug to prevent vascular diseases by reducing metabolic risk factors. However, from the clinic there is a justifiable question; should hormone therapy be continued beyond management of menopausal symptoms? With the current knowledge the answer is no, because the beneficial effects do not outweigh the adverse side effects. Side effects however could be minimized by local treatment. In this thesis we tested whether changes in lipid and glucose metabolism are induced by a direct effect of E_2 on liver. This seems to be true for regulation of glucose- but not for lipid metabolism (**chapter 3 & 4)**. In agreement with the fact that the effect on lipid metabolism takes a significant time to develop, it suggests an indirect effect of E_2 on lipid metabolism. Therefore, it seems unlikely that E_2 will become serious competition for the commonly available and effective lipid-lowering drugs. Alternatively, the beneficial effect of E_2 on

hepatic insulin sensitivity obtained within six hours after treatment is interesting and deserves further study. Moreover, the simultaneously induced peripheral insulin resistance implies the importance of tissue specific modulation.

Locally in the vessel wall, our data indicate that E_2 and ER subtype specific ligands form an attractive drug to prevent in-stent restenosis after PTCA. In the clinic, introduction of drug eluting stents has led to a tremendous reduction of in-stent restenosis. E_2 has been shown to prevent restenosis (chapter 8) and in comparison to alternative drugs which solely reduce VSMC proliferation, E_2 has also been shown to promote re-endothelialization [30]. However, we have not addressed this issue in our model and this requires further study. For the improvement of therapeuty, a thorough understanding of the effects of E_2 and the interplay between ERα and ERβ in the vasculature is required. By use of ER subtype specific ligands, we have demonstrated that both ERα and ERβ are involved in the vascular protective effects of E_2. A future challenge will be to determine to what degree the ERα versus ERβ are involved during the different stages of injury during the development of the restenotic process as well as during the development of atherosclerosis. Thus, selective pharmacological targeting of ER subtypes may represent a novel and promising approach in the treatment of in-stent restenosis.

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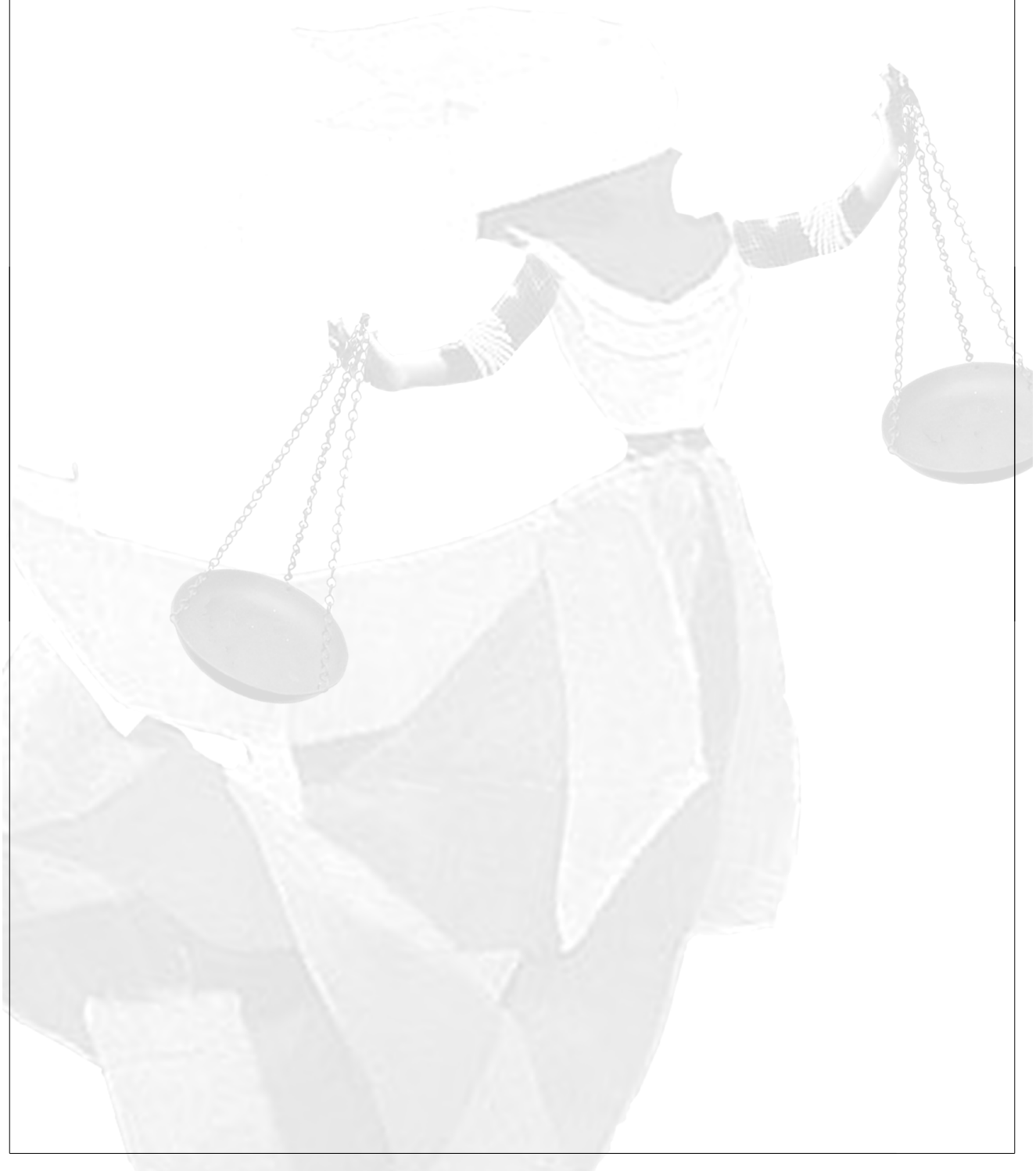
10.

Samenvatting



Samenvatting

Hart- en vaatziekten vormen de belangrijkste doodsoorzaak in de westerse wereld. Voor een groot deel is dit het gevolg van een sterke vernauwing van de bloedvaten (aderverkalking), bekend onder de term “atherosclerose”. Bij deze aandoening vormen zich zogenaamde plaques (opeenhopingen van cholesterol en cel materiaal) onder de beschermende laag van endotheelcellen aan de binnenkant van bloedvaten. De voornaamste factoren die de kans op het ontstaan van atherosclerose verhogen zijn weinig beweging, hoge bloeddruk, een hoge bloedsuiker spiegel en een hoog cholesterol gehalte.

Atherosclerose blijkt veel minder voor te komen in vrouwen voor de menopauze, in vergelijking met mannen van dezelfde leeftijd. Opmerkelijk is dat na de menopauze de incidentie van atherosclerose in vrouwen sterk stijgt. Deze stijging loopt parallel met metabole veranderingen, zoals gewichtsstijging, een verandering van de vetstofwisseling en verhoogde bloedsuikerspiegels. Kortom, na de menopauze ontstaat er een ongunstig risicofactor profiel. Deze observaties hebben tot de suggestie geleid dat oestradiol, het vrouwelijke sekshormoon, bescherming kan bieden tegen het ontstaan van atherosclerose.

Dierstudies hebben een direct bewijs geleverd voor de beschermende rol van oestradiol in het ontstaan van atherosclerose. In atherosclerose gevoelige muismodellen leidt toediening van oestradiol tot minder grote plaques [1-4]. Ondanks deze klaarblijkelijke positieve effecten moet men voorzichtig zijn met het gebruik van oestradiol als preventief geneesmiddel. Oestradiol kan namelijk ook nadelige processen activeren. Zo verhoogt het de kans op borstkanker, baarmoederkanker en galstenen. Bovendien leveren humane studies met betrekking tot het effect van toediening van extra oestradiol na de menopauze tegenstrijdige resultaten op [5-8]. Kortom, de uiteindelijke effecten van oestradiol op de vaatwand is complex en nog niet helemaal helder.

Om de nadelige effecten te vermijden en de voordelige effecten van oestradiol uit te buiten, is het van belang om precies te weten wat oestradiol wel en niet doet én in welk weefsel. De organen die in dit proefschrift de aandacht hebben gekregen, zijn vaatwand en lever. De vaatwand; door zijn directe betrokkenheid bij de ontwikkeling van atherosclerose. De lever; omdat dit orgaan een centrale rol speelt bij het op peil houden van de bloedsuikerspiegel en vet stofwisseling. En zoals hierboven beschreven, wanneer de bloedsuikerspiegel en vet stofwisseling verstoord zijn vormt dit een risico voor het ontstaan van atherosclerose. Beide organen, lever en vaatwand zijn ook potentiële doelwit organen van

oestradiol. Zij kunnen namelijk het in het bloed circulerende oestradiol herkennen doordat zij een specifieke receptor, de oestradiol receptor (ER) bevatten. De ER is een receptor die aanwezig is in de cel, welke na binding van oestradiol geactiveerd wordt. Een geactiveerde ER kan vele processen in de cel beïnvloeden. Tot dusver zijn er twee verschillende ER types bekend, ERα en ERβ. Dit maakt de beoogde effecten van oestradiol complex. ERα en ERβ kunnen namelijk verschillende processen activeren, soms zelfs leidend tot tegenovergestelde effecten. Daarom is het van belang om de ERα en ERβ routes van elkaar te kunnen onderscheiden. Zeker in het geval wanneer beide receptoren aanwezig zijn, zal het specifiek moduleren van ofwel ERα ofwel ERβ meer inzicht geven dan wanneer je oestradiol niveaus verandert en dus beide aanzet.

In **hoofdstuk 2 $^{t}/_{m}$ 4** van dit proefschrift werd onderzocht of de lever een belangrijke rol speelt bij de effecten van oestradiol op de lipiden en glucose huishouding. Allereerst, is er in **hoofdstuk 2** een nieuwe techniek opgezet, genaamd 'RNA interference' (RNAi) om de ERα signaleringsroute uit te zetten. Deze techniek berust op het feit dat kleine, sequentie specifieke moleculen, short hairpin (sh)RNAs genaamd, binden aan een homologe sequentie om deze vervolgens af te breken. In verschillende cellijnen tonen we aan dat onze shRNAs gemaakt tegen de muis ERα (shERα) werkzaam zijn, ze verminderen de ERα activiteit met 80%. Vervolgens is dit shERα construct in een adenovirale (Ad) vector gezet. Deze Ad vector dient louter als transport vehikel om in de cel zijn bagage (lees shERα moleculen) af te leveren. Een bijkomend voordeel is dat Ad vectoren in de muis erg efficiënt en exclusief lever cellen infecteren, een van onze doelwit organen. Na injectie van Ad.shERα, laten we zien dat ERα niveaus en activiteit in de lever significant lager zijn. Kortom we laten zien dat Ad vectoren effectief zijn om shERα moleculen naar de lever te brengen, om daar het ERα gen af te breken.

De studie beschreven in **hoofdstuk 3** had ten doel om de functie van ERα in lever te bestuderen wat betreft het reguleren van lipid parameters. Met behulp van Ad.shERα was 60% van de ERα transcripten in de lever afgebroken. Desondanks waren de lipid parameters in bloed en lever en ook de glucose waardes niet veranderd. Deze resultaten laten zien dat ERα niveaus in de lever niet bepalend zijn om lipid parameters te reguleren.

Oestradiol lijkt ook betrokken te zijn bij glucose metabolisme en insuline gevoeligheid. Na de menopauze zijn er meer vrouwen die ongevoelig zijn voor insuline. Toevoeging van oestradiol lijkt geassocieerd met het verbeteren van insuline gevoeligheid. Maar, omdat de voordelige effecten van oestradiol op insuline gevoeligheid gepaard gaan met

een verandering in lichaamsgewicht, is de rol van oestradiol op het reguleren van de glucose huishouding onduidelijk. Om meer inzicht te krijgen in de directe capaciteiten van oestradiol in glucose metabolisme ten behoeve van insuline gevoeligheid, hebben wij in **Hoofdstuk 4** de onmiddellijke effecten van oestradiol op de lever insuline gevoeligheid bepaald. Muizen die ongevoelig zijn voor insuline zijn behandeld met oestradiol. Vervolgens is zes uur na oestradiol toediening de insuline gevoeligheid bepaald met behulp van een gevoelige techniek. Onze data laten zien dat in de muizen die met oestradiol waren behandeld de glucose productie sterk geremd wordt gedurende de hoog insuline conditie. Deze data impliceren een belangrijke rol voor oestradiol in het verbeteren van de insuline gevoeligheid van de lever.

Kort samengevat, deze studies waarin de oestradiol signalering route kort wordt veranderd, laten zien dat de lever een belangrijke rol speelt in het glucose metabolisme, maar niet in het lipid metabolisme. De voordelige effecten, zoals verminderde zwaarlijvigheid (obesitas) en ophoping van lipiden in lever en in het plasma die gevonden zijn na een langdurende oestradiol behandeling [9-12], zullen hoogst waarschijnlijk geïnduceerd zijn door effecten in andere organen en weefsels. Het zou ook goed mogelijk kunnen zijn dat de veranderingen in de lipid waarden een indirect gevolg zijn van de al snel door oestradiol geïnduceerde veranderingen op insuline gevoeligheid. Het is immers bekend dat regulatie van vet en glucose metabolisme met elkaar geassocieerd zijn.

Naast de voordelige effecten die oestradiol blijkt te hebben op het metabolisme, zijn er ook aanwijzingen die suggereren dat oestradiol een positieve rol speelt in de vaatwand. Zowel ERα als ERβ zijn aanwezig in de cellen die de vaatwand bekleedt [13-18]. Maar er zijn nog veel onduidelijkheden over de effecten van oestradiol op de vaatwand. Oorzaak hiervoor is het feit dat de effecten complexer zijn dan in de lever. Je hebt in de vaatwand namelijk te maken met verschillende cel types die zowel ERα als ERβ heeft, terwijl de lever alleen ERα bevat. Bovendien zijn er geen *in vivo* (muis) modellen beschikbaar waarin lokaal ERα en ERβ gemoduleerd zijn. Met de studies uitgevoerd in hoofdstukken **5** $^{t}/_{m}$ **8**, wilden we meer inzicht krijgen in de rol van ERα en ERβ in de vaatwand. Om dit doel te bereiken hebben we constructen gemaakt waarmee de ER specifiek en op een fysiologische manier in de vaatwand kan worden veranderd.

Studies op geïsoleerde cellen (zogenaamde in vitro studies), zijn de makkelijkste manier om weefsel specifieke effecten te bestuderen. Het introduceren van genetisch materiaal zoals shERα of de ER zelf, is de manier om specifiek de functie van dit gen te onderzoeken. Helaas zijn de meeste vasculaire cellen moeilijk te transfecteren en infecteren. In **hoofdstuk 5** hebben we dit probleem aangepakt door de natuurlijke voorkeur van Ad vectoren te veranderen. Normaal gesproken komen Ad vectoren efficiënt de cel in doordat zij de coxsackie virus Ad receptor (CAR) herkennen die op het oppervlak van de desbetreffende cel zit. Helaas hebben vasculaire cellen vrijwel geen CAR op hun oppervlak. Daardoor zijn zij dus moeilijk te transfecteren. Om Ad toch als transport vehikel te kunnen gebruiken hebben we een 'dubbelzijdig plakband' construct gemaakt. Dit construct bestaat uit het Ad bindende domein van CAR aan de ene kant, en aan de andere kant een RGD peptide. Dit resulteert in een targeting construct wat de Ad vector bindt en affiniteit heeft voor integrines. We laten zien dat dit "targeting" construct in staat is om transfectie efficiëntie naar zowel endotheel als vasculaire spier cellen aanzienlijk te verhogen. Omdat dit targeting construct efficiënt werkt en te gebruiken is voor vrijwel elke willekeurige Ad vector, is dit systeem bijzonder geschikt om de functie van een gen in vasculaire cellen in vitro te onderzoeken.

Vervolgens hebben we bepaald of ons getarget virus ook toepasbaar is in een in vivo situatie. Ons doelwit orgaan was de halsslagader (carotis arterie) van de muis (**hoofdstuk 6**). Dit is een stuk moeilijker. Allereerst wordt transport naar de vaatwand belemmerd doordat Ad vectoren normaliter door de lever worden weg gevangen. Met ons construct hebben we deze eerste barrière overwonnen. Ad vectoren die gebonden waren aan CAR-cRGD werden niet opgenomen door de lever. Ondanks deze lever "de-targeting", zagen we geen opname in de normale of zelfs beschadigde vaatwand. Dit kan verklaard kunnen worden doordat de Ad vector erg snel uit de bloedbaan was opgeruimd. Bovendien ligt de halsslagader niet op de meest toegankelijke positie en vormt het onbeschadigde niet-delende endotheel wellicht een ontoegankelijke barrière. Om deze barrières te omzeilen hebben we de Ad vector +/- cRGD, voor 10 minuten lokaal in de beschadigde halsslagader gebracht. Maar zelfs onder deze condities was het niet mogelijk om de genen de vaatwand in te transporteren. Mogelijkerwijs is het partikel te groot, en/of komt de dynamiek van integrine expressie niet overeen met de korte tijdsduur dat het virus aanwezig is. Deze studie samen met een aantal andere studies waarin het niet gelukt is om Ad vectoren naar vaatcellen in het levende dier te sturen, geeft aan dat ondanks het feit dat we veel van Ad vectoren weten, er toch nog essentiële kennis mist betreffende de regulatie van Ad opname in vivo.

Hoofdstuk 5 & 6 samenvattend, laten we zien dat gen overdracht naar vaatcellen via Ad vectoren in vitro goed mogelijk is. Alleen zijn er nog onbekende factoren in de in vivo situatie die gen overdracht voorkomen.

In **hoofdstuk 7**, hebben we het effect van oestradiol op de expressie van adhesie moleculen in endotheel cellen geanalyseerd. Een verhoogde aanwezigheid van leukocyt adhesie moleculen op het endotheel is een van de eerste reacties van het vat op schade. Deze adhesie moleculen zorgen ervoor dat ontstekingscellen naar het beschadigde gebied komen. Uit onze studie blijkt dat wanneer je oestradiol toedient voordat de expressie van adhesie factoren wordt geïnduceerd, deze expressie significant geremd wordt. Dus, oestradiol lijkt de ontstekingsreactie tegen te gaan. Verder hebben we onderzocht wat de rol van ERα niveaus is op dit oestradiol geïnduceerde effect. Het rationele voor deze studie is het feit dat ERα minder aanwezig is in een plaque in vergelijking tot 'normale' vaten en vaten waarin aderverkalking nog in een vroege fase is [16,19,20]. De vraag is of de actie van oestradiol verminderd is doordat er minder ERα aanwezig is. In de endotheel cellen hebben we de ERα niveaus en activiteit met 60% verminderd. Maar dit leidde niet tot een verandering in de respons op oestradiol. Oestradiol zorgde nog steeds voor een verminderde expressie van adhesie moleculen. Echter, wanneer we de ERα activiteit geheel uitschakelden, werd het effect van oestradiol wel geheel geblokkeerd. Deze data laten zien dat ERα noodzakelijk is voor het ontstekingsremmende effect van oestradiol, maar dat de hoeveelheid van de ERα niveaus geen bepalende factor is.

In vivo zijn zowel ERα als ERβ aanwezig in de vaatwand. Omdat deze verschillende effecten kunnen induceren, zouden de uiteenopende resultaten na oestradiol behandeling verklaard kunnen worden door een verschil in hoeveelheid en balans tussen ERα en ERβ. In **hoofdstuk 8**, hebben we de rol van beide receptoren in de vaatwand bestudeerd. Het proces waar we ons op hebben gericht heet neointima vorming. Neointima vorming wordt gekarakteriseerd door een continue deling van vasculaire gladde spiercellen die een vernauwing van het bloedvat tot gevolg heeft. In hoofdstuk 8, hebben we neointima geïnduceerd door om het bloedvat in het been van de muis (femoral arterie) een kleine cilindervormige plastic buis (cuff) te plaatsen. Daarbij is de cuff zo ontwikkeld dat je het kan vullen met geneesmiddelen die vervolgens ter plekke en gelijktijdig met het induceren van de neointima in de vaatwand vrijkomen. Op deze manier kun je testen of de geneesmiddelen

lokaal de groei van gladde spiercellen kan remmen. Om specifiek de rol van ERα en ERβ op neointima vorming te onderzoeken hebben we de cuffs geladen met oestradiol of ERα en ERβ specifieke activatoren. Onze data laten zien dat ERα en ERβ andere, maar niet per se totaal tegenovergestelde routes induceren. De meest interessante bevinding is dat ERβ aanwezig in de vaatwand een beschermende rol kan bieden tegen het ontstaan van neointima. Deze beschermende rol van ERβ was dusver onbekend. Dit bevestigt dat onderzoek naar weefsel en ERα en ERβ specifieke effecten van belang is.

Samengevat, kunnen we concluderen dat het nuttig is om modellen en constructen te creëren die het mogelijk maken om weefsel en ER specifieke effecten te bestuderen. Tot dusver hebben wij aangetoond dat ERα in de lever geen sterke rol speelt in het reguleren van lipid parameters, maar wel in het reguleren van glucose productie. In de vaatwand lijkt het erop dat naast ERα ook ERβ neointima vorming tegen kan gaan.

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Abbreviations

AAV	Adeno-Associated Viruses
ACCα	Acetyl CoA Carboxylase α
ACO	Acetyl CoA Oxidase
Ad	Adenoviruses
Ad-shERα	Adenovirus mediated expression of shRNA against mouse ERα
AF	Activation function
ApoE	Apolipoprotein E
ArKO	Aromatase deficient mice
BGU	Body Glucose Uptake
Bio-cRGD	Biotinylated cyclic RGD peptide
BrdU	5-bromo-2'-deoxyuridine
CAR	Coxsackie adenovirus receptor
CCD	Charged coupled device
CHO	Chinese Hamster Ovary
Chol	Cholesterol
CMV	Cytomegalovirus
cRGD-Ad	cRGD targeted Ad-vector
CYP7A1	cholesterol 7α-hydroxylase
CYP8B1	sterol 12α- hydroxylase
DBD	DNA Binding Domain
DMEM	Dulbecco's modified Eagle's medium
E_2	17-β-estradiol
EC	Endothelial cells
ECM	Extracellular matrix
eNOS	endothelial Nitric-Oxide Synthase
EPCs	Endothelial progenitor cells
ER	Estrogen Receptor
EREs	Estrogen Response Elements
$ER\alpha^{-/-}$	ERα knockout
$ER\beta^{-/-}$	ERβ knockout
$ER\alpha/\beta^{-/-}$	Double ER knockout mice
FAS	Fatty Acid Synthase
FFA	Free Fatty Acid
FGF	Fibroblast growth factor
GFP	Green Fluorescent Protein
G6P	Glucose-6-Phosphatase
GP	Glycogen Phosphorylase
HGP	Hepatic Glucose Production
HL	Hepatic lipase
HPS	Hematoxylin-phloxine-saffron
HRT	Hormone Replacement Therapy
HSP90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
Ip	Intraperitoneal
LacZ	β-Galactosidase
LBD	Ligand-Binding Domain
LDL	Low-density lipoprotein
Ldlr	Low-density lipoprotein receptor
Luc	Luciferase
M	Mouse
MOI	Multiplicity of Infection
NERKI	Non-classical ER Knock-In
NO	Nitric Oxide
ovx	ovariectomy
PEPCK	Phospho Enol Pyruvate Carboxy Kinase
Pfu	Plaque Forming Unit
PGC-1α	Peroxisomal Proliferators-Activated Receptor-γ coactivator 1α
PTCA	Percutaneous transluminal coronary angioplasty
S.c	Subcutaneous
RISC	RNA-induced silencing complex
RNAi	RNA interference
SHP	short heterodimer partner
shRNA	short hairpin ds RNA
shERα	shRNA specific for mouse ERα
siRNA	small interfering RNA
SREBP1c	Sterol Regulatory Element-Binding Protein-1c
TG	triglyceride
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WHI	Women's Health Initiative
Wt	Wild type

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Krom YD, Gras JC, Frants RR, Havekes LM, Berkel TJ, Biessen EA, Willems van Dijk K. Efficient targeting of adenoviral vectors to integrin positive vascular cells utilizing a CAR-cyclic RGD linker protein. Biochem Biophys Res Commun. 2005 Dec 16;338(2):847-54.

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Curriculum Vitae

Yvonne Krom werd geboren op 4 maart 1978 te Akersloot. Na het behalen van het VWO diploma aan het PCC te Alkmaar in 1996, begon zij in datzelfde jaar met de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. In het jaar 1997 werd het propedeutische examen behaald. Tijdens de doctoraal fase liep zij een gecombineerde stage bij de vakgroepen Immunologie en Gen Therapie van de Vrije Universiteit onder supervisie van Dr. H. Haisma. Onderwerp van de stage was het genereren en karakteriseren van een monoklonaal antilichaam tegen luciferase. De tweede stage werd verricht onder leiding van Dr. C.C. Hoogenraad bij de afdeling Cel biologie en Anatomie aan de Erasmus Universiteit te Rotterdam. Tijdens deze stage werd onderzoek verricht naar de in vivo lokalisatie van CLIP-170, een cytoplasmatisch linker eiwit. In 2000 behaalde zij het doctoraal examen Medische Biologie aan de Vrije Universiteit Leiden.

Van september 2000 tot januari 2005 was zij werkzaam als assistent in opleiding (AIO) op de afdelingen Humane Genetica van het Leids Universitair Medisch Centrum, onder leiding van Dr. K. Willems van Dijk, en op het Gaubius Laboratorium, TNO-Preventie en Gezondheid onder leiding van Prof. Dr. Ir. L.M. Havekes, beide te Leiden. De resultaten van dit door NWO gesubsidieerde onderzoek staan beschreven in dit proefschrift.

Vanaf september 2005 is zij werkzaam als postdoc op de afdeling Humane Genetica van het Leids Universitair Medisch Centrum onder leiding van Prof. Dr. R.R. Frants en Prof. Dr. Ir. S.M. van der Maarel. Hier probeert zij meer duidelijkheid te verschaffen over de epigenetische invloed in de spierziekte genaamd, facioscapulohumeral muscular dystrophy (FSHD).